CHARACTERIZATION OF THE MECHANISM OF THE SUB-TYPE SELECTIVE, SLOW-DISSOCIATION OF A CARBOSTYRIL BASED β-ADRENERGIC RECEPTOR AGONIST FROM THE β_2-ADRENERGIC RECEPTOR

By

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TABLE OF CONTENTS

ACKNOWLEDGMENTS ii

LIST OF TABLES vi

LIST OF FIGURES vii

ABSTRACT ix

CHAPTER 1 INTRODUCTION 1

Overview of the β-Adrenergic Receptor 1
Activation of the β-Adrenergic Receptor 3
Structure of the β-Adrenergic Receptor and the Family of GPCRs 11
Study of the Function of the Hydrophilic Extracellular Portion of the Receptor 12
Study of the Function of the Hydrophilic Intracellular Portion of the Receptor 15
Study of the Function of the Hydrophobic Transmembrane Domain of the Receptor 17
Molecular Modeling of GPCRs 24
β_2-Selective Agonists 33
Development of Short-Acting Agonists 33
Background of Long-Acting or Slow-Dissociating β_2AR Ligands 35
Carbostyril-Based Long-Acting β_2AR Selective Agonists 38
Specific Aims of the Current Project 43

CHAPTER 2 MATERIALS AND METHODS 51

Materials 51
Methods 52
Receptor Nomenclature 52
Mutagenesis 54
Cell Culture and Transfections 55
Membrane Preparations and Pretreatments 55
Saturation Radioligand Binding Assays 56
Competition Binding Experiments 57

Cellular Localization of Expressed Receptors 57
Measurement of cAMP Accumulation 57
Pretreatment of DDT Cells to Remove Receptor Reserve 58
Determination of Apparent Ligand Dissociation Rates 59
Localization of Measured cAMP 59
Phosphodiesterase Activity 60
Data Analysis 60
Chemical Synthesis 61
Analytical Procedures 61
Synthetic Procedures 61

CHAPTER 3 FUNCTIONAL CHARACTERIZATION OF THE SERINE^{165} TO ALANINE HUMAN β_2-ADRENERGIC RECEPTOR MUTANT 75

Introduction 75
Methods 77
Confirmation of Transfected Receptor Genotype using Reverse Transcriptase-Polymerase Chain Reaction 77
Membrane Pretreatment with *N*-Ethylmaleimide 77
Results 78
Discussion 81

CHAPTER 4 CHARACTERIZATION OF THE CARBOSTYRIL NUCLEUS BINDING SITE WITHIN THE β_2-ADRENERGIC RECEPTOR USING SITE DIRECTED MUTAGENESIS 95

Introduction 95
Results 97
Discussion 100

CHAPTER 5 STRUCTURE-ACTIVITY RELATIONSHIPS OF SLOW DISSOCIATING CARBOSTYRIL-BASED AGONISTS FROM THE β_2ADRENERGIC RECEPTOR 108

Introduction 108
Results 108
Discussion 114

CHAPTER 6 CHARACTERIZATION OF THE STRUCTURAL REQUIREMENTS OF THE SUBTYPE SELECTIVE SLOW DISSOCIATION KINETICS USING CHIMERIC β_1/β_2 ADRENERGIC RECEPTOR 128

Introduction 128

Results 131
Discussion 135

CHAPTER 7 MOLECULAR MODELING OF THE COMPLEX FOMED BETWEEN THE CARBOSTYRIL-BASED AGONIST **1** AND THE HUMAN β_2-ADRENERGIC RECEPTOR 146

Introduction 146
Methods 147
Results 150
Discussion 153

CHAPTER 8 CONCLUSIONS AND FUTURE DIRECTIONS 160

LITERATURE CITED 170

BIOGRAPHICAL SKETCH 193

LIST OF TABLES

Table page

2-1. Generalized numbering scheme for GPCR sequences 53

3-1. Ligand binding parameters of WT and $S^{4.57(165)}A$ β_2-ARs 80

3-2. EC50 values and maximal stimulation of agonist stimulated cAMP accumulation in cells expressing WT and $S^{4.57(165)}A$ β_2-ARs 81

3-3. Results of two-state model analysis 86

4-1. Equilibrium binding and adenylyl cyclase stimulation parameters for ISO and carbostyril 1, 7, and 8 for murine L cells expressing WT and mutant β_2ARs 97

5-1. Agonist properties of β-agonists 110

5-2. Agonist apparent k_{off} and K_i values 113

6-1. Dissociation rates of carbostyril **1** and ISO from βAR constructs 131

6-2. Radioligand binding data 133

7-1. Amino acid sequence of the TM domains used in the Swiss Protein automated model builder 147

7.2. The important inter- and intra-helical contact points maintained during construction of starting structures 148

7.3. Amino acid sequence of the TM domains used in the final β_2AR model 148

LIST OF FIGURES

Figure page

1-1. Helical net representation of the human β_2-AR 45

1-2. The carbostyril ligands employed in studies of the insurmountable agonist activity selective for the β_2-AR 46

1-3. Effect of carbostyril **1** on cAMP accumulation if DDT and C6 cells....47

1-4. Effect of carbostyril **1** on βAR in C6 and DDT cell membranes 48

1-5. Effect of carbostyril **1** on the cAMP accumulation in transfected mouse L cells 49

1-6. Effect of carbostryil **1** on the βAR in transfected mouse L cell membranes 50

3-1. Confirmation of the $S^{4.57(165)}A$ mutation using RT-PCR and restriction endonuclease analysis 88

3-2. Localization of the expression of the β_2-AR with intact cell binding....89

3-3. Agonist competition with [^{125}I]-CYP for WT and $S^{4.57(165)}A$ β_2-ARs in the absence and presence of guanine nucleotide....90

3-4. Effect of pretreatment with (-)-ISO, Gpp(NH)p and/or NEM on [125I]-CYP binding to membranes containing either the WT or $S^{4.57(165)}A$ β_2-ARs....91

3-5. Agonist-stimulated cAMP accumulation in cells expressing either the WT or $S^{4.57(165)}A$ β_2-ARs 92

3-6. The two-state model of receptor activation 93

3-7. Theoretical stimulation curves based on two-state mathematical model....94

4-1. Chemical structures of carbostyril-based β-agonists **1**, **3-6** and ISO 104

4-2. Synthetic scheme of carbostyril **7** and **8** 105

4-3. Effect of serine mutations on the binding of ISO and carbostyril **1** 106

4-4. Competition binding and functional characteristics of site-directed mutants of the β_2AR 107

5-1. Synthetic scheme for 8-hydroxycarbostyril derivatives 122

5-2. Synthetic scheme for catecholamine derivative 123

5-3. Relationship between βAR occupancy and maximal response in DDT cells 124

5-4. Time course of cAMP accumulation and decline in DDT cells 125

5-5. Linear regression analysis of the relationship between the apparent k_{off} and K_I 126

5-6. Effect of β-agonist pretreatment on βAR content of DDT cell membranes 127

6-1. Schematic representation of chimeric receptors 140

6-2. [^{125}I]-CYP radioligand competition data for carbostyril **1** and ISO using murine L cells expressing WT β_1 and β_2ARs, and β_2 / β_1AR chimeras 141

6-3. [^{125}I]-CYP radioligand competition data for carbostyril **1** and ISO using murine L cells expressing β_2 / β_1AR TM IV chimeras 142

6-4. Apparent dissociation behavior of carbostyril **1** at the WT β_1AR and the WT β_2AR 143

6-5. Apparent dissociation behavior of carbostyril **1** and ISO at β_1/β_2AR chimeras 144

6-6. Apparent dissociation behavior of carbostyril **1** at β_1 and β_2AR TM IV chimeras 145

7-1. The model of the complex between the β_2AR and carbostyril **1** 156

7-2. Carbostyril **1** bound within the agonist-binding site of the β_2AR 157

7-3. The interaction of $Arg^{3.50(131)}$ with the conserved interhelical hydrogen bond between $Asp^{2.50(79)}$ and $Asn^{7.49(322)}$ 158

7-4. Interaction of carbostyril **1** with the agonist binding site and adjacent non-conserved structures in TM IV 159

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The β-adrenergic receptors (βARs) are members of a supergene family of G-protein coupled receptors. They are structurally characterized by the presence of seven hydrophobic transmembrane (TM) domains that span the plasma membrane. Three subtypes of the βARs have been described. The β_1 and β_2ARs have been extensively studied and pharmacologically differ in their affinities for norepinephrine and epinephrine. A novel series of carbostyril-based βAR agonists has identified additional differences. Many carbostyril derivatives are potent β-agonists that bind and activate both the β_1 and β_2ARs with similar potencies and efficacies. However, several carbostyril-based β-agonists tightly bind to and slowly dissociate from the β_2AR. In the present study, the structural features of both the β_2AR and the 8-hydroxy-5-[2-[(1-phenyl-2-methylprop-2-yl)amino]-1-hydroxyethyl]-carbostyril **1** which contribute to its

dissociation and binding properties at the β_2AR were investigated. The binding site of carbostyril **1** has been elucidated using complementary mutations in the β_2AR and the ligand. The binding site is analogous to the catecholamine-binding site. Specifically, the charged amine function of the side chain forms an electrostatic interaction with $Asp^{3.32(113)}$ and the 8-hydroxy moiety forms a hydrogen bond with $Ser^{5.47(207)}$. The binding selectivity has been mapped as a function of the side chain substituents using a series of carbostyril derivatives. The data show that the 8-hydroxycarbostyril nucleus and the positioning of the side-chain geminal methyls and phenyl groups are important characteristics determining the high affinity and slow dissociation of carbostyril **1** from the β_2AR. The specific TM domain of the β_2AR that confers subtype selective binding of carbostyril **1** was recognized using a series of β_1/β_2AR chimeras. Specifically, the data suggests that residues 158-173 in TM IV of the β_2AR are required for the novel antagonist-insensitive pseudo-irreversible binding of carbostyril **1**. Molecular modeling, which has been used to rationalize the data and to generate a generalized understanding of the slow dissociation of carbostyril **1** from the β_2AR, has identified possible interactions between the side chain of carbostyril **1** and TM VII. However, no direct interactions were identified with residues of TM IV suggesting that differences in the amino acid sequence of TM IV may affect the dynamics of the β_2AR to change the dissociation behavior of carbostyril **1**.

CHAPTER 1
INTRODUCTION

Overview of the β-Adrenergic Receptor

The β-adrenergic receptors (βARs) are members of the superfamily of genes that encode for cell surface receptors that regulate the activity of GTP-binding regulatory proteins (G-proteins). These cell surface receptors couple to G-proteins for transmembrane signaling. G-protein coupled receptors (GPCRs) are important in the regulation of various aspects of cellular activity. This receptor superfamily contains several hundred distinct proteins capable of responding to approximately 80 % of the known hormones and neurotransmitters (Birnbaumer *et al.*, 1990; Schofield and Abbott, 1989; Birdsall, 1991; Mollon, 1991). The responses to these various hormones and neurotransmitters leads to many basic physiologic effects such as contraction of smooth and striated muscles, gland excretion, induction of complex behavior (affection, depression, aggression), vision, taste and smell (Cronet *et al.*, 1993). Many of these important physiological functions are mediated by effectors such as the transmission of neural signals through synapses, regulation of phospholipid turnover, controlling the exchange of K^+ and Ca^{++} through the plasma membrane, and the regulation of enzyme activity and gene transcription through the modulation of second messenger concentrations. Some of the receptors identified that are involved in these physiological functions are the family of odorant and taste (Lancet, 1991a; Lancet, 1991b), muscarinic

acetylcholine (Curtis *et al.*, 1989), serotonergic (Hartig, 1989), dopaminergic (Sibley, 1991), histaminergic (Birdsall, 1991), and the adrenergic receptors (Lefkowitz and Caron, 1988).

The α and βARs mediate the physiologic effects of catecholamines. Currently, molecular cloning have shown the existence of at least three separate mammalian βARs designated β_1, β_2 and β_3ARs (Frielle *et al.*, 1987; Dixon *et al.*, 1986; Emorine *et al.*, 1989) and in all cases, recombinant βARs expressed in various mammalian cells display identical pharmacological profiles to their cognate native expressed receptors, as assessed from cell or tissue studies. The β_1 and β_2ARs differ pharmacologically in their affinity for the endogenous catecholamine agonist norepinephrine (NE) and for various synthetic antagonists (Stiles and Lefkowitz, 1984). Principally, the β-receptors were subdivided into the β_1- and β_2-subtypes because epinephrine (Epi) and NE are essentially equipotent at the β_1AR, whereas, Epi is 10- to 50-fold more potent at the β_2AR (Lands *et al.*, 1967). The β_3AR differs in its affinity for Epi, NE and (-)-isoproterenol (ISO) in that ISO and NE are equipotent, with both being more potent than Epi. Interestingly, most β-antagonists, including propranolol, don't block the β_3AR (Lefkowitz *et al.*, 1990).

There is differential expression and physiologic function of these βAR subtypes. The β_1AR is primarily expressed in heart and regulates the ionotropic, chronotropic and dromotropic effects of NE and Epi, and secondarily in the juxtaglomerular cells in the kidney to modulate renin secretion. The β_2AR is responsible for a much broader range of effects due to its wide tissue distribution. Stimulation of the β_2AR leads to the relaxation of arterioles, veins and the muscles of the bronchioles and uterus. It also leads to the

decrease in stomach and intestine motility. These effects are primarily due to the expression of the β_2AR in smooth muscle cells. The β_2AR is expressed in the liver and stimulates glycogenolysis and gluconeogenesis. Glycogenolysis is also stimulated in skeletal muscle and is regulated by the expression of the β_2AR (Lefkowitz *et al.*, 1990). Additionally, the β_2AR can increase the secretion of insulin and has many affects in the central nervous system (Mayer, 1990). The β_3AR was originally cloned (Emorine *et al.*, 1989) as an atypical βAR because of its unusual pharmacology (Lefkowitz *et al.*, 1990), however, it is expressed in adipocytes and stimulation results in increased lipolysis of triglycerides. Additional atypical pharmacology was found in certain tissues, which may be due to the expression of a β_4AR. A recent review has proposed the existence of a β_4AR subtype to explain unusual pharmacological activities in the mammalian heart. The pharmacology of the putative β_4AR is distinct from the β_1, β_2 and β_3ARs (Molenaar *et al.*, 1997).

Activation of the β-adrenergic receptor

A significant portion of our knowledge on the mechanisms of G-protein coupled receptor processes has come from the study of the βARs. The βARs are coupled to the stimulatory guanine nucleotide binding protein (G_S) that regulates the activity of a membrane associated enzyme adenylyl cyclase (AC) (Smigel *et al.*, 1984). General agreement now exists that the regulation of AC activity involves at least three proteins including the receptor, G_S and the catalytic unit of the enzyme, AC (Stiles *et al.*, 1984; Levitzki *et al.*, 1993).

There are two prevailing theories concerning the mechanism of G-protein, G_s, activation by the GPCR, β_2AR that leads to the activation of the second messenger systems such as AC. These two theories can be summarized by the ternary complex model and the two-receptor states model. The ternary complex model proposes that the initial activation step is the formation of a ternary complex consisting of the agonist, receptor, and G_S. The binding of the agonist in the ternary complex is of high affinity. This is followed by GTP binding to G_S resulting in a destabilization of the complex leading to a reduction in agonist affinity (increased dissociation) and G_S activating the catalytic unit. Termination of enzyme activity involves the hydrolysis of GTP to GDP by the α-subunit of G_S and the cycle of activation can begin again (Stiles *et al.*, 1984; Levitzki *et al.*, 1993). The current two receptor states model proposes that the receptor alone is in constant equilibrium between an active, R*, and an inactive, R, conformation of the receptor (Leff, 1995; Lefkowitz *et al.*, 1993). The R* conformation of the receptor can interact with the G-protein, such as G_s, to stimulate the dissociation of GDP and allow the binding of GTP to G_s, which eventually leads to the activation of AC. The basal amount of cAMP formed in the absence of agonist is due to the equilibrium constant between R and R* conformations and the interaction of the transient R* conformation with G_s. With a larger population of R*, greater amounts of cAMP are produced through the activation of G_s and AC. Therefore, any shift in the equilibrium constant will result in a change in the amount of cAMP produced by AC. The role of full agonists in the two-receptor states model is to shift the equilibrium toward the R* conformation through mass action by having a much higher affinity for the R* conformation. Partial agonists have a

higher affinity for R* but still significantly bind to R to cause a reduction in the maximal efficacy observed. Neutral antagonists have equal affinity for the R and R* conformations which only serves to block the action of agonists and inverse agonists. Inverse agonists are thought to have a higher affinity for the R conformation and serve to shift the equilibrium toward the R conformation to reduce the basal amount of cAMP produced by AC. The two-receptor states model was evoked to explain the phenomenon of constitutively active receptors and inverse receptor agonists.

The discovery of constitutively active receptors was important for the current understanding of the molecular processes involved in activation of G-protein-coupled receptors. Prior to this discovery, the ternary complex model was sufficient to explain many of the salient features of ligand binding to the β_2AR (DeLean *et al.*, 1980). There were three observations that lead to the model that equates the active form of the receptor with a ternary complex involving the agonist, the receptor and the G-protein. The first observation was that agonists, but not antagonists, were able to distinguish between a high- and low-affinity state of the receptor. Additionally, the proportion and relative affinity of ligands for these two receptor affinity states was proportional to the intrinsic efficacy of the agonist. Finally, guanine nucleotides are able to shift the receptor to the low-affinity state by actively uncoupling the G-protein from the ternary complex. From these findings the ternary complex model stated that agonists stabilize and increase the formation of the high-affinity ternary complex. Full agonists are the best at stabilizing the ternary complex and lead to the shallow displacement curves in competition binding experiments. These curves obtained with full agonists become right-shifted the greatest

with the addition of guanine nucleotides. Partial agonists are intermediate in their ability to stabilize the ternary complex and result in less shallow displacement curves that are less right-shifted in the presence of guanine nucleotides. Antagonists block the binding of agonist and therefore prevent the formation of the ternary complex. Additionally, because activation of the receptor is dependent on the presence of the agonist in the ternary complex, inverse efficacy of antagonists was theoretically not allowed. This fact illustrates a key feature of the ternary complex model. The model requires the presence of G-protein for the high-affinity agonist binding state of the receptor and the presence of agonist for receptor activation (Kent *et al.*, 1980; DeLean *et al.*, 1980; Lefkowitz *et al.*, 1993; Leff, 1995).

It has been shown that many GPCRs when transfected in heterologous cell expression systems can activate intracellular messenger systems without the addition of agonist (Allen *et al.*, 1991; Ren *et al.*, 1993; Samama *et al.*, 1993; Barker *et al.*, 1994; Chidiac *et al.*, 1994; Tiberi and Caron, 1994; Costa *et al.*, 1992). Additionally, this tendency can be increased dramatically in certain recombinant receptor mutants (Cotecchia *et al.*, 1990; Kjelberg *et al.*, 1992; Samama *et al.*, 1993; Ren *et al.*, 1993; Allen *et al.*, 1991; Robinson *et al.*, 1992; Robbins *et al.*, 1993; Parma *et al.*, 1993; Shenker *et al.*, 1993; Rao *et al.*, 1994; Nanevicz *et al.*, 1996; Burstein *et al.*, 1996; Lefkowitz *et al.*, 1993). Lefkowitz and co-workers discovered that the interchange of a discrete homologous region of amino acids located in the carboxyl-terminal portion of the third intracellular loop of the G_q-phospholipase C-coupled α_{1B}AR and the G_s-adenylyl cyclase-coupled β_2AR results in constitutively active receptors. In both cases the basal or

agonist-independent intracellular signaling activity was nearly identical to that of the fully activated agonist-stimulated WT receptors (Cotecchia *et al.*, 1990; Samama *et al.*, 1993; Ren *et al.*, 1993). Interestingly, the affinity for agonists at the constitutively active receptors was dramatically higher, although, there was no change in the affinity for antagonist. Additionally, compounds with the highest intrinsic efficacy had the greatest shift in affinity at the constitutively active receptors as compared to WT. These changes in the affinity were observed in systems that were deficient in G-proteins and were not affected by the addition of guanine nucleotides (Samama *et al.*, 1993).

The fact that agonists display increases in affinity for constitutively active mutant receptors that are proportional to their intrinsic efficacy in the absence of G-protein coupling forced a reevaluation of the ternary complex model. It now appears that the efficacy of a compound is determined by receptor binding alone. In light of this data, a two-state (Leff, 1995) or an allosteric ternary complex (Lefkowitz *et al.*, 1993) model was proposed for the activation of GPCRs and, ultimately, the downstream second messenger systems. This model introduces a structural isomerization event that regulates the formation of the R* state, which can interact with G-proteins. In the case of constitutively active receptors, an increase in the isomerization constant results in a greater proportion of the receptors in the R* state, which results in the abnormally high basal second messenger activity. WT receptors normally have very low basal activity due to the small proportion of receptors in the R* state (Lefkowitz *et al.*, 1993). Therefore, the role of agonists is to displace the equilibrium one way or the other upon binding (Leff, 1995). In this way, agonist efficacy can be simplified to be proportional to the relative affinities for the R and/or R* states. If an agonist has a much greater affinity for

the R* state as opposed to the R state, then it is considered a full agonist. A partial agonist would have a higher affinity for the R* state but would have some measurable affinity for the R state. Neutral antagonists have an equal affinity for both the R and R* states and cause no shift in the equilibrium between the states. Finally, a new class of compounds, not considered under the ternary complex model, that have a higher affinity for the R state and would disfavor the formation of the R* state. These compounds have been called inverse agonist and can dramatically reduce the agonist-independent activity observed with constitutively active mutant receptors. The degree in which these compounds decrease the basal activity is dependent on its inverse efficacy in a manner analogous to that observed for full and partial agonists (Leff, 1995).

To further delineate the molecular mechanisms of drug action at GPCRs, Gether *et al.* (1995) studies the changes in fluorescence when ligands of various efficacies bind to a fluorescent-labeled, purified WT β_2AR. Fluorescent labels incorporated into proteins have been shown to be sensitive indicators of conformational changes and of protein-protein interactions that cause changes in polarity of the environment surrounding the probe (Cerione, 1994; Phillips and Cerione, 1991; Dunn and Raftery, 1993; Gettins *et al.*, 1993). Therefore, purified β_2AR was labeled with the cysteine-specific and environmentally sensitive fluorescent probe N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylene-diamine (IANBD). The addition of full agonists and strong partial agonists to the fluorescent-labeled β_2AR resulted in decreases in fluorescent emissions in a stereospecific, dose-dependent and reversible manner. The decreases were not observed for weak partial agonists or antagonists. However, inverse

agonists caused a small but measurable increase in the fluorescence emissions, which is consistent with antagonists causing conformational changes in the receptor. The overall magnitude of the fluorescence changes correlated inversely with the intrinsic efficacy of the ligand studied (Gether *et al.*, 1995).

Additional data supporting the hypothesis of ligand induced conformational changes affect receptor activation was obtained by characterizing the structural instability and ligand-induced fluorescent changes of a constitutively active β_2AR mutant (Gether *et al.*, 1997). This study characterized the changes in fluorescence emissions of a fluorescent-labeled purified constitutively active mutant of the β_2AR as well as the structural stability in the presence and absence of ligands with a range of intrinsic efficacies. It was shown that the constitutively active receptor mutant had two novel properties. These are that they are structurally instable and exhibit an exaggerated conformational response to ligand binding as compared to the WT β_2AR. A common mechanism resulting in these two novel properties of the constitutively active receptor mutant was suggested by the authors. They suggested that it can be explained if the thermodynamic barrier for the inter-conversion between the R and R* states is lowered to allow rapid transitions and if the isomerization constant is increased to elevate the proportion of receptors in the R* state for the constitutively active receptor mutant. Additionally, the R* state can be considered a high-energy, intermediate state that can be stabilized by G-protein and/or agonist. Without G-protein or agonist present, the expected half-life for R* would be short due to its high energy. The fraction of receptors n the R* state at any given time in the absence of G-protein of agonist would be small. The R*

state would rapidly return to the ground state, R, or physically denature. The structural instability observed for the constitutively active receptor mutant as compared to the WT β_2AR is due to a larger fraction of the receptor population occupying the R* state. With this increased population, a greater number of receptors denature as compared to the WT β_2AR. Therefore, any compound that can either stabilize the R* state, agonists, or reduce the amount of the R* state, inverse agonists, increased the overall stability of the constitutively active receptor mutant by reducing the possibility for physical denaturation from the R* state (Gether *et al.*, 1997).

Another observation by Gether *et al.* (1997) was that the agonist-induced florescent changes were enhanced at the constitutively active mutant of the β_2AR. This effect was shown to be a result of the smaller thermodynamic barrier to the R* state from the R state. Therefore, a greater fraction of the receptors convert to the R* state following agonist occupancy. Additionally, the small increases in the fluorescent emissions following the binding of an inverse agonist to the WT β_2AR were greatly increased in the fluorescent-labeled constitutively active receptor mutant. This indicated that the thermodynamic barrier for the conversion of R from R* is similarly reduced (Gether *et al.*, 1997).

The current two-state receptor model describes intrinsic conformation changes that lead to the activation of GPCRs and their respective second messenger systems (Lefkowitz *et al.*, 1993; Leff, 1995). Recent studies that have investigated the receptor conformational changes as a result of ligand binding, have shown that they can be correlated with intrinsic efficacy (Gether *et al.*, 1995; Gether *et al.*, 1997). Therefore, it is

important to understand the intimate structural features of GPCRs that may lead to changes in the isomerization constant and in the agonist-induced disruptions in the equilibrium between the inactive R state and the active R* state. An understanding of these parameters will aid in our ability to understand the molecular events required for receptor activation.

Structure of the β-Adrenergic Receptor and the Family of GPCRs

It has been validated through biochemical, immunological, and biophysical approaches that a consensus architecture exists for all GPCRs. This basic architecture (Figure 1-1) consists of an extracellular amino-terminus, a cytoplasmic carboxyl-terminus, and a transmembrane domain consisting of seven hydrophobic helical segments, which pass through the membrane, connected by three extracellular and three intracellular hydrophilic loops (Ballesteros and Weinstein, 1995). The amino acid sequence of each transmembrane segment contains a distinct pattern of two or three conserved residues (Baldwin *et al.*, 1997). The hypothesis that this structural class of cellular receptors possesses seven α-helical segments passing through the membrane was developed by analyzing patterns of residue hydrophobicity (Ballesteros and Weinstein, 1995). Subsequently, the hypothesis was experimentally confirmed by electron cryomicroscopy studies of bacteriorhodopsin (Henderson *et al.*, 1990) and, more recently, rhodopsin (Schertler *et al.*, 1993; Schertler and Hargrave, 1995; Unger and Schertler, 1995; Unger *et al.*, 1997). This experimental work has also yielded information on the packing of these helical segments within the membrane.

The primary structures of the βARs have been elucidated by the molecular cloning of their genes and/or cDNAs. The βARs are members of and are structurally related to this superfamily of genes that encode for GPCRs. Given their medical importance, several regions of the βAR and other representatives of the GPCR family have been mutagenized. The effects of these mutations on the function of these receptors have been assessed. This data has been used to further define the details of the molecular structures and of the mechanisms of receptor activation. These studies can be categorized into three groups of studies. These studies can be grouped into those that study the role of residues found in the amino-terminus and the extracellular loops, in the carboxyl-terminus and intracellular loops and in the transmembrane segments.

Study of the Function of the Hydrophilic Extracellular Portion of the Receptor

The extracellular hydrophobic portion of the receptor is comprised of three extracellular loops and the amino-terminal region. With large peptide ligands, the extracellular portion appears to be important for ligand recognition, but with small molecules, it appears to be the TM domain. In either case, the extracellular domains are important for constraining the dynamics of the receptor for a biologically active receptor. Several investigators have attempted to characterize the function of this extracellular portion of the receptor.

Conserved cysteine residues found in the exofacial loops of the β_2AR, conserved throughout all known GPCRs, seem to be critical for maintaining a biologically active receptor (Strosberg, 1990; Ostrowski *et al.*, 1992; Fraser *et al.*, 1989). The second and third extracellular loop regions each contain a highly conserved cysteine residue. An

analysis of rhodopsin has revealed that these residues are involved in an intramolecular disulfide bond to link these two extracellular loop regions (Karnik *et al.*, 1988; Davidson *et al.*, 1994). Specifically, site-directed mutagenesis of either of these cysteine residues to serine in bovine rhodopsin gave proteins that were expressed at reduced levels, glycosylated abnormally, and unable to bind 11-cis-retinal. Therefore, Cys-110 and Cys-187 in bovine rhodopsin are essential for the correct tertiary structure of the protein. (Karnik *et al.*, 1988). Additionally, it was shown by Davidson *et al.* (1994) that if either of these cysteines are mutated to alanine the stability of the metarhodopsin II photointermediate is greatly reduced and leads to decreased coupling efficiency with transducin. Site-directed mutagenesis of either conserved cysteine to a valine in the β_2AR results in a destabilization of the tertiary structure of these receptors. The mutants also have significant alterations in the ligand binding properties of the receptors. Therefore, it has been suggested that these residues are involved in maintaining the active conformation of these receptors (Dixon *et al.*, 1987; Dohlman *et al.*, 1990).

Additionally, the destabilization of the receptor that is observed by mutagenesis of these important cysteine residues can also be repeated by reducing the receptor with dithiothreitiol (DTT). Treatment of the β_2AR with DTT has been shown to abolish ligand binding to the receptor. However, if the receptor is pretreated with the ligand prior to reduction, then the loss of binding is prevented. It was hypothesized that the ligand blocked the DTT reactive site located in the receptor. Subsequently, Lin *et al.* (1996) showed that the binding of the ligand stabilized the tertiary structure of the receptor without changing the ability of DTT to reduce the disulfide bond.

Furthermore, cysteine residues 190 and 191 in the second extracellular loop of the human β_2AR are required for either maintenance of a receptor conformation that allows for high affinity ligand binding possibly through a vicinal disulfide bond (Fraser, 1989). It has been shown in another family of receptors, the ligand-gated ion channels, that a vicinal disulfide bond in the extracellular domain of the nicotinic acetylcholine receptor is important for activation by acetylcholine (Kao *et al.*, 1984; Mishina *et al.*, 1985; Kao and Karlin, 1986; Mosckovitz and Gershoni, 1988).

GPCRs are glycosylated with heterogeneous patterns of glycosylation. At least a single consensus sequence for N-linked glycosylation (Asn-X-Ser/Thr) exists in the extracellular domain of all receptors cloned to date. These consensus sequences are usually located in the N-terminal portion of the molecule. These putative N-linked glycosylation site have been shown to be glycosylated with site-directed mutagenesis of the β_2AR, the NK1 neurokinin receptor, the lutropin receptor and rhodopsin (Rands *et al.*, 1990; Kage *et al.*, 1991; Fong *et al.*, 1992; Liu *et al.*, 1993; Kaushal *et al.*, 1994). The role of the N-linked glycosylation appears to be different in different receptors. In the β_2AR, preventing glycosylation does not appear to alter ligand binding, coupling to GS or stimulation of AC. However, the non-glycosylated receptors were less abundant at the cell surface (Rands *et al.*, 1990). Glycosylation of the NK-1 receptors is not important for high affinity peptide binding (Fong *et al.*, 1992). Additionally, non-glycosylated lutropin receptors are able to bind human choriogonadotrophin with high affinity, but are not expressed on the cell surface (Liu *et al.*, 1993) Finally, N-linked glycosylation of

rhodopsin is important for signal transduction but does not mediate ligand binding or cell surface expression in COS-1 cells (Kaushal *et al.*, 1994).

Study of the function of the hydrophilic intracellular portion of the receptor

The intracellular hydrophilic portion of the receptor contains three intracellular loops and the carboxyl-terminus. These regions have been proposed to be important for the intracellular signaling of the receptor. Segments of the third intracellular loop have been implicated in the interaction of the βAR with G_S (Ostrowski *et al.*, 1992). While regions in the C-terminal tail appear to be important for receptor desensitization (Dohlman *et al.*, 1991; Strosberg, 1990; Ostrowski *et al.*, 1992).

Early mutagenesis studies using 19 mutant receptors comprising substitutions and deletions of native sequence in the cytoplasmic domains of the β_2AR, indicated that the second intracellular loop region and conserved cysteine residues were important for maintaining the proper orientation of the G-protein binding domain of the receptor. Most importantly, the carboxyl-terminal portion of the third intracellular loop and the amino-terminal portion of the carboxyl-tail were shown critically important for G-protein coupling to the receptor (O'Dowd *et al.*, 1988). A deletion of a discrete region of the third intracellular loop of the β_2AR (D267-273) resulted in a reduction in the ability of the mutant to stimulate adenylyl cyclase. This reduction in the efficacy of the receptor was not accompanied by a reduction in the high-affinity agonist binding or with changes in agonist-induced receptor sequestration (Hausdorff *et al.*, 1990). This appears to be an additional example of a separation in the structural determinates which confer high-affinity agonist binding and the ability of the receptor to stimulate adenylyl cyclase.

Although this discrete region of the β_2AR doesn't mediate receptor sequestration, an adjacent domain (residues 259-262) in the third intracellular loop region appears to be the consensus region for receptor phosphorylation by cAMP-dependent protein kinase (Clark *et al.*, 1989). Most recently, Burstein *et al.* (1996) have identified two residues (Ile^{216} and Thr^{220}) of the amino-terminal portion of the third intracellular loop of the m5 muscarinic receptor that form the structural foundation for the G-protein binding pocket. Within this binding pocket, Tyr^{217} and Arg^{223} were shown to form specific interactions with the G-protein. Therefore, it is believed that G-proteins are recruited by specific ionic interactions with the receptor and are activated by hydrophobic residues located on the third intracellular loop of the receptor. The activation of the G-protein by these hydrophobic residues is suggested by the authors because they found that if Thr^{220} is mutated to cysteine or a valine, the receptor shows a modest degree of constitutive activity (Burstein *et al.*, 1996). Mutation in this region of the β_2AR have also lead to constitutive activation of this receptor (Samama *et al.*, 1993).

A conserved cysteine located in the C-terminal tail of many GPCRs has been shown to be palmitoylated in the α and βARs (O'Dowd *et al.*, 1989; Kennedy and Limbird, 1993), and rhodopsin (Papac *et al.*, 1992). Palmitoylation in this position may allow portion of the C-terminal tail to be anchored in the membrane to functionally create a fourth intracellular loop and to possibly constrain the tertiary structure of the C-terminus (Strader *et al.*, 1994). However, anchoring is not required for receptor function, because when palmitoylation is prevented through site-directed mutagenesis of the cysteine residue to a serine, leucine or alanine in rhodopsin (Karnik *et al.*, 1988), the

β_2AR (Dixon *et al.*, 1987) or the α_{2A}AR (Kennedy and Limbird, 1993), respectively, the overall structure or function is unchanged. Interestingly however, if the cysteine residue is changed to a glycine residue in the βAR, the receptor is unable to activate G-protein.

An additional role for the carboxyl-terminal segment of rhodopsin has been suggested by studying protein-sorting pathway of rhodopsin in the presence of a monoclonal antibody and Fab fragments to the carboxyl-terminal segment. The monoclonal antibody and Fab fragments are able to strongly inhibit vesicle formation. Additionally, they are able to arrest newly synthesized rhodopsin in the trans-golgi network and not in the golgi. Therefore, it appears that the carboxyl-terminal segment of rhodopsin mediates the sorting of rhodopsin at the exit of the trans-golgi network through an interaction with the intracellular sorting machinery (Deretic *et al.*, 1996).

Study of the function of the hydrophobic transmembrane domain of the receptor

Mutagenesis and chimeric receptor studies have shown that the hydrophobic segments of adrenergic receptors form the ligand binding pocket (Kobilka, 1992; Ostrowski *et al.*, 1992). In addition to this region providing amino acid side chains that specifically interact with ligands, it has been shown that micro-domains exist in the TM region. These micro-domains are critically involved in switching the receptor from an inactive state to the active state (Ballesteros *et al.*, 1998; Sealfon *et al.*, 1995).

Ligand binding site. Upon cloning of the βAR, structural similarities to rhodopsin were noted. Therefore, it was suggested that the conserved transmembrane domains of the receptors might serve as a common site of agonist binding (Dixon *et al.*, 1986). Subsequently, experimental evidence for this hypothesis was obtained when it was shown

that the extramembraneous regions of the receptor could be deleted without affecting the ability of agonists or antagonists to bind (Dixon *et al.*, 1987). Deletion mutants of the transmembrane regions to narrow the amino acids involved in ligand recognition were not successful. Therefore, an approach was taken where single amino acids were changed in the TM segments to assess their role in agonist or antagonist binding. The ability to work with recombinant β_2ARs in site-directed mutagenesis studies (Ostrowski *et al.*, 1992; Strader *et al.*, 1989c) has provided detailed molecular insights into the structural features important for catecholamine binding. Many of the molecular interactions with a putative binding site can be deduced from the functionality of the catecholamine agonists. These functional groups provide the binding specificity of the agonist within the binding pocket. Therefore, functional correlates must exist in the agonist-binding site of the receptor. All catecholamine ligands, such as NE and Epi, have a protonated amine located on the side chain. It was suggested that there should be a negatively charged counter ion in the agonist-binding site. This lead to the identification of $Asp^{3.32(113)}$ in TM III as interacting with the protonated side chain amino group of β-agonists through salt bridge formation (Strader *et al.*, 1988). This residue is completely conserved throughout the family of GPCRs interacting with biogenic amine ligands. The validity of this assignment was confirmed by complementary mutagenesis of the receptor and ligand to restore the functional response through an alternate interaction. Specifically, the mutation of $Asp^{3.32(113)}$ to a serine abolishes receptor activation by biogenic amines. However, modification of the ligand amino group to an ester or ketone gave compounds capable of full activation of the mutant β_2AR, presumably through a hydrogen-bonding interaction.

The restoration of full activity with the complementary serine mutation at this receptor locus with an ester or a ketone on the ligand provides strong evidence for the spatial alignment of the aspartate residue of the WT receptor and the protonated amine of catecholamines (Strader *et al.*, 1991).

The catechol hydroxyl groups present on the β-agonists suggest receptor side chain hydrogen bond donor acceptor functionality, similar to that found on serine residues. In similar studies involving site-directed mutagenesis with ligand complementation, it was also shown that $Ser^{5.43(204)}$ and $Ser^{5.46(207)}$ in TM V hydrogen bond to the *meta*- and *para*- phenolic groups of the catechol moiety, respectively (Strader *et al.*, 1989b). In addition, $Phe^{6.51(289)}$ in TM VI probably interacts with the aromatic ring of the ligand through dispersion interactions (Strader *et al.*, 1989a; Strader *et al.*, 1989b). Additional mutagenesis experiments, in combination with limited molecular modeling studies, indicate that $Phe^{6.51(289)}$, together with $Trp^{6.48(286)}$ and $Phe^{6.52(290)}$ probably constitute a hydrophobic pocket in the β_2AR (Dixon *et al.*, 1988; Strader *et al.*, 1994).

The role of other residues in the β_2AR has also been investigated using site-directed mutagenesis. An important goal of these studies has been to define the specific interaction between the β-hydroxyl group of β-agonists and the β_2AR (Strader *et al.*, 1989a; Mitchell *et al.*, 1989). $Ser^{4.57(165)}$ is a completely conserved residue among adrenergic receptors and was an early candidate as the hydrogen bond donor/acceptor for the β-hydroxyl group of β-agonists (Trumpp-Kallmeyer *et al.*, 1992). Early attempts by others, to characterize a mutant β_2AR in which $Ser^{4.57(165)}$ is replaced by alanine were unsuccessful, presumably due to failures in expressing active protein (Strader *et al.*,

1989a). More recently, it has been shown that $Ser^{4.57(165)}$ plays no role in the binding of catecholamine agonists (Wieland *et al.*, 1996; Hehr *et al.*, 1997). Wieland *et al.* (1996) clearly demonstrated that $Asn^{6.55(293)}$ in TM VI of the β_2AR interacts directly with the β-hydroxyl function of βAR agonists and is responsible for the stereoselective binding of these agonists.

Studies aimed at delineating the structural properties that yield agonist and antagonist subtype selectivity for the β_1 and β_2ARs have employed chimeric receptors (Frielle *et al.*, 1988; Dixon *et al.*, 1989; Marull *et al.*, 1990). These proteins comprise a well-defined mixture of the TM helices and loops from both receptors. The pharmacological properties of these chimeric proteins, when expressed in *Xenopus laevis* oocytes indicated that TM region IV was principally responsible for determining the relative affinities of Epi and NE for the β_1 and β_2ARs. In contrast, TM segments VI and VII appeared to play primary roles in controlling antagonist selectivity (Frielle *et al.*, 1988). While these experiments showed that other TM regions were involved in modulating ligand selectivity, their contributions were considerably less important. In contrast, however, to the results on β-agonist selectivity obtained using chimeric β_1/β_2ARs, TM VII was observed to be a major determinant for both agonist and antagonist selectivity in experiments employing the chimeric α_2/β_2ARs. Additional experiments using chimeric β_1/β_2ARs have implicated TM VII in mediating the high affinity binding of the carbostyril-based agonist, TA-2005 to the β_2AR (Kikkawa *et al.*, 1997).

Similar experiments have been extended to studies of amino acids defining the binding site for biogenic amines in the α-adrenergic (Wang *et al.*, 1991), dopaminergic (Pollock *et al.*, 1992; Mansour *et al.*, 1992), histaminergic (Gantz *et al.*, 1992), muscarinic (Fraser *et al.*, 1989; Wess *et al.*, 1991) and serotonin (Shih *et al.*, 1991; Ho *et al.*, 1992; Almaula *et al.*, 1996a; Almaula *et al.*, 1996b) receptors. The results taken collectively are consistent with the hypothesis that the ligand-binding pocket for biogenic amines is located within the TM regions of these GPCRs (Strosberg, 1990; Ostrowski *et al.*, 1992; Fraser *et al.*, 1989; Mitchell *et al.*, 1989; Lewell, 1992). It is reasonable to assume a common binding site for small molecule ligands throughout the subfamily of GPCRs (Strader *et al.*, 1994), because retinal is bound in a similar position in rhodopsin relative to the membrane (Baldwin *et al.*, 1997). Although peptidyl-ligand receptors, such as the opiate receptors, have been shown to use the extracellular loops for ligand recognition, a portion of the peptide-ligand binds within the TM segments to stimulate the receptor (Strahs and Weinstein, 1997). Therefore, the agonist-binding site within the TM segments may be a common feature to the entire superfamily of GPCRs.

Regions involved in receptor activation. Both the ternary complex model and the two-state receptor model require that conformational changes occur in the receptor that lead to the activation of the G-protein. In the ternary complex model, these conformational changes are induced by an agonist. The two-state model states that the conformational changes occur in rapid equilibrium and that agonists stabilize the active form of the receptor to shift the equilibrium toward a larger population of the active form of the receptor. In either case, the binding of an agonist to the TM segments leads to

distant conformational changes in the intracellular loops that allow for G-protein coupling and activation. Specific intramolecular interactions in the TM segment appear to be critical for this signal transduction.

Early clues that ligand binding and the guanine nucleotide sensitivity of agonist binding could be dissociated from the ability of the receptor to maximally stimulate adenylyl cyclase were obtained using a mutant of the β_2AR in which $Cys^{6.47(285)}$ was changed to a serine. This mutation did not affect ligand binding or the receptors sensitivity to guanine nucleotide to affect the agonist affinity. However, this mutant had an impaired ability to stimulate cAMP formation in the presence of ISO (Fraser, 1989).

Additionally, residues at loci 2.50 and 7.49 are highly conserved and are an Asp and an Asn, respectively, in all GPCRs except for the gonadotrophin-releasing hormone (GnRH) receptor. In the GnRH receptor, these conserved residues are interchanged so that there is an Asn and an Asp at loci 2.50 and 7.49, respectively. The combination of the conserved nature of these residues and the reciprocal changes seen with the GnRH receptor suggest that these residues are required for function of GPCRs (Zhou *et al.*, 1994). This hypothesis was verified in the 5-HT_{2A}-serotonin receptor (Sealfon *et al.*, 1995). In these experiments, the aspartate at locus 2.50 and the asparagine at locus 7.49 were mutated to alanine. These mutations did not cause a change in the binding properties of agonist, however, they did abolish the serotonin (5-HT)-stimulated increases in phosphatidylinositol (PI) hydrolysis. These same results were obtained using a mutant receptor construct in which the aspartate at locus 2.50 was mutated to an asparagine. Interestingly, the mutant receptor in which the asparagine at locus 7.49 was changed to an

aspartate showed no change in either the agonist binding affinity or the maximal 5-HT-stimulated PI hydrolysis. However, a moderate increase was observed in the concentration of 5-HT which produces 50 % of maximal PI hydrolysis (EC_{50}) as compared to the WT receptor. The combination of mutations that structurally reverses the position of the Asp and Asn residues at loci 2.50 and 7.49 ($D^{2.50}N$, $N^{7.49}D$), resulted in a partial rescue of the maximal 5-HT-stimulated PI hydrolysis observed with the single $D^{2.50}N$ mutant. The authors hypothesized that the existence of a hydrogen-bonding network between TM segment II and VII is required for receptor activation, but not agonist affinity (Sealfon *et al.*, 1995).

This hydrogen-bonding network was recently extended following mutagenesis experiments involving the activation of the GnRH receptor. It appears that there are three residues located in TM segments II, III, and VII that form a hydrogen-bonding network in the active conformation of the receptor (Ballesteros *et al.*, 1998). Using a combination of molecular modeling and saturation mutagenesis, a conserved arginine-cage motif was found to exist in TM III of the GnRH receptor that acts a molecular switch between the inactive and active states of the receptor. In the inactive state of the receptor, the conserved arginine residue at locus 3.50 forms a hydrogen bond to the similarly conserved aspartate residue at locus 3.49. During activation of the receptor, $Asp^{3.49}$ becomes protonated and releases the hydrogen bond with $Asn^{3.50}$. The side chain is guided to form a hydrogen bond with the side chain of $Asn^{2.50}$ ($Asp^{7.49}$ in the GnRH receptor) by the bulky side chain of the conserved $Ile^{3.54}$ (Ballesteros *et al.*, 1998). In the αAR, the mutation of $Asp^{3.49}$ to an Asn leads to constitutive activation of the receptor (Scheer *et al.*,

1996; Scheer *et al.*, 1997). Also consistent with this model, a $Glu^{3.49}$ to Gln mutation in rhodopsin leads to similar activity (Cohen *et al.*, 1993). Additionally, $Glu^{3.49}$ has been shown to take up a proton upon activation of the receptor (Arnis *et al.*, 1994).

Other conserved amino acid residues found in the adrenergic receptor family may be important for the activation of the β_2AR. $Ser^{4.57(165)}$ is an example of a completely conserved residue among the adrenergic receptors in which the functional role remains unanswered. However, a clue to its role in the signal transduction of the β_2AR came with the identification of a β_2AR polymorphism found expressed in patients with asthma. This polymorphism, in which $Thr^{4.56(164)}$ is mutated to isoleucine, was functionally characterized using site-directed mutagenesis (Green *et al.*, 1993). The $T^{4.56(164)}I$ β_2AR mutant, expressed in CHW-1102 cells, exhibited lower ligand binding affinity with ISO and NE, but not with β-agonists lacking a β-hydroxyl group. Moreover, in agonist competition experiments conducted in the absence of GTP, the $T^{4.56(164)}I$ β_2AR mutant failed to exhibit detectable high affinity binding. There was also a 50% reduction in basal and agonist stimulated AC activity, consistent with a failure to form an agonist/receptor/G_s ternary complex. Therefore, there may be evidence supporting the hypothesis that $Ser^{4.57(165)}$ is important for ternary complex formation, and activation of the receptor following agonist binding. If this hypothesis is correct, and given the apparent functional role of $Thr^{4.56(164)}$, it appears, that TM segment IV of the β_2AR may play an important role in receptor activation.

Molecular Modeling of the GPCRs

In the absence of structural data, and given the likely structural similarities of seven transmembrane helical GPCRs seen from sequence alignment studies (Kervalage, 1991; Baldwin, 1993; Baldwin *et al.*, 1997), homology modeling methods have been widely employed to obtain structural models for the TM segments of these cellular receptors (Mitchell *et al.*, 1989; Cronet *et al.*, 1993; Trumpp-Kallmeyer *et al.*, 1992; Maloney-Huss and Lybrand, 1992; Ballesteros and Weinstein, 1995; Strahs and Weinstein, 1997; Donnelly *et al.*, 1994; Luo *et al.*, 1994; Alkorta and Du, 1994; Perlman *et al.*, 1994; Hibert *et al.*, 1991; Findlay and Eliopoulos, 1990; Lewell, 1992; Ijzerman *et al.*, 1992; Peroutka, 1991; Evans *et al.*, 1991). There is now widespread use of such models that have proven value in hypothesis generation, the interpretation of receptor structure-function relationships and in the design of ligands with which to verify specific ligand-receptor interactions (Donnelly *et al.*, 1994; Strahs and Weinstein, 1997; Ballesteros and Weinstein, 1995).

Given its biomedical importance, the extensive range of agonists and antagonists, and the availability of site-specific mutants, the β_2AR has been the subject of a number of previous modeling studies (Mitchell *et al.*, 1989; Lewell, 1992; Maloney-Huss and Lybrand, 1992; Donnelly *et al.*, 1994; Trumpp-Kallmeyer *et al.*, 1992; Cronet *et al.*, 1993; Hibert *et al.*, 1991). While early structural models of the β_2AR were built using the high-resolution structure of bacteriorhodopsin (Henderson *et al.*, 1990), the validity of such structures is questionable given that this bacterial protein is a proton pump and is not coupled to G proteins (Pardo *et al.*, 1992). Consequently, studies that are more recent have employed the helix packing observed in the low-resolution experiments on

rhodopsin (Schertler *et al.*, 1993; Schertler and Hargrave, 1995; Unger and Schertler, 1995; Unger *et al.*, 1997). After almost a decade of work, the major issues in developing useful structural models of the transmembrane segments of GPCRs have been identified (Baldwin *et al.*, 1997), and various strategies now exist for solving this complex molecular modeling problem (Ballesteros and Weinstein, 1995). In particular, methods for identifying amino acids within the TM segments have been investigated in detail (Edelman, 1993). It is likely that that the boundaries of the TM helices are defined by charged residues, such as arginine and lysine, especially at junctions with the intracellular loops (Ballesteros and Weinstein, 1992; Donnelly *et al.*, 1994). Analysis of the periodicity of side chain properties has also been a major contribution in identifying residues defining α-helical secondary structures in membrane proteins (Donnelly *et al.*, 1994). In the case of the β_2AR, it is increasingly likely that the amino acids in the TM segments have been correctly assigned (Donnelly *et al.*, 1994; Ballesteros and Weinstein, 1995). Recent elegant studies using α_2/β_2AR chimeras have established that the helices are arranged in an anti-clockwise orientation as viewed from the extracellular surface of the plasma membrane (Mizobe *et al.*, 1996). In separate experiments, residues $Leu^{7.37(310)}$, $Leu^{7.38(311)}$ and $Asn^{7.39(312)}$ in TM segment VII, possessing the β_2AR sequence were mutated to the cognate residues in the α_2AR ($L^{7.37(310)}F$, $L^{7.38(311)}F$ and $N^{7.39(312)}F$), resulting in the production of a non-functional receptor. Function could be restored, however, by making complementary mutations in TM segments I-III and VI, which accommodated the bulky side chains of the phenylalanine substituents. The restoration of function was only

consistent with these TM regions surrounding TM VII, as required by an anti-clockwise orientation.

Even given this information on directionality, the helix-helix contacts must be defined together with the residues that are not located in the lipids of the membrane. Most investigators agree that the side chains of conserved amino acids, especially those that are charged or hydrophilic, are placed within the cavity defined by the seven helices, as these residues are involved in making specific interactions with agonists. Highly variable, hydrophobic residues in sequence alignments of known GPCRs are thought to interact with lipid.

Without direct structural data, there have been three major indirect approaches taken to verify the hypothesis concerning the specific interhelical contact points that determine the orientation of the helices with regard to one another. The first approach, was the use of complementary mutations that restore functional properties to mutational inactivated receptors (Suryanarayana *et al.*, 1992). The second approach attempted to introduce metal-ion-binding sites as probes for helix-helix interactions (Elling *et al.*, 1997). Finally, Oprian and co-workers have used an elegant cysteine cross-linking approach in split receptors to demonstrate the proximity of two residues located in adjacent helices (Yu *et al.*, 1995).

The cumulative information gained using these techniques is summarized below and is thought to be extrapolated to at least the family of rhodopsin-like GPCRs (Elling *et al.*, 1997). There are specific interactions proposed in the adrenergic receptors between a specific amino acid in the seventh hydrophobic segment and a discrete region of the first transmembrane segment (Suryanarayana *et al.*, 1992). Two highly conserved residues

among all GPCRs are located in helix II and VII and have been shown to form a specific ionic bond that is required for either receptor-G-protein coupling or high-affinity agonist binding in the serotonin 5-HT_{2A} receptor or the gonadotrophin-releasing hormone receptor, respectively (Sealfon *et al.*, 1995; Zhou *et al.*, 1994). Additionally, it was shown using complementary mutagenesis that TM segment I and II form a specific interaction that is required for pharmacological activity in the m2 and m5 muscarinic receptors (Liu *et al.*, 1995). Biophysical and mutational studies of constitutively active forms of rhodopsin have shown that $Lys^{7.49(296)}$ in TM VII, which forms the Schiff base with retinal, is located in close proximity to a glutamate residue in TM III ($Glu^{3.49(113)}$) and a glycine residue in TM II ($Gly^{2.50(90)}$) (Robinson *et al.*, 1992; Rao *et al.*, 1994).

Using zinc-site engineering, it was originally shown that there is a specific orientation between transmembrane helix V and VI in the NK-1 receptor (Elling *et al.*, 1995). These results were extended to possibly the entire family of rhodopsin-like GPCRs when an analogous zinc-binding site was created in the κ-opioid receptor (Thirstrup *et al.*, 1996). Additional evidence that these two helices are orientated similarly in the entire family of rhodopsin-like family of GPCRs was generated using a cysteine cross-linking study of split receptor constructs of rhodopsin. Oprian and co-workers verified that the specific interaction between the TM segments V and VI shown to exist in the NK-1 and κ-opioid receptors exists in rhodopsin (Yu *et al.*, 1995).

Finally, a systematic attempt to probe helix-helix interactions in the NK-1 receptor using zinc-site engineering has identified two additional interhelical interactions. There appears to be a glutamate residue in TM V (Glu^{193}) in close proximity to an

asparagine residue in TM III (Asn^{109}). This interaction appears to be across the agonist-binding site of the NK-1 receptor. A second interaction was identified in this study, it appears that the naturally occurring histidine residue in TM III (His^{108}) and a tyrosine residue in TM II (Tyr^{92}) are in close proximity (Elling and Schwartz, 1996).

A significant body of evidence now exists to support the hypothesis that GPCRs are structurally related to rhodopsin and not bacteriorhodopsin. A systematic analysis of 204 GPCR sequences has suggested that the helical arrangement of GPCRs more closely resembles the projection map of rhodopsin. In this study, each helix was arranged relative to one another based upon the extent of its lipid-facing surface area. The sequence analysis indicated that helices I, IV, and V are most exposed to the lipid environment of the membrane, helices II, VI, and VII have an intermediate exposure to the lipid, and helix III is significantly isolated from most lipid. Assuming an ordered arrangement of the helices due to short interhelical loops, there were only two possible arrangements. These arrangements were clockwise orientation with respect to the extracellular face and an anti-clockwise orientation. The anti-clockwise orientation was chosen because of relative interhelical distances found to be important for the function of rhodopsin. For rhodopsin, the retinal chromophore is covalently attached to $Lys^{7.49(296)}$. The resulting positively charged Schiff base requires a negative counter ion. The counter ion was identified as $Glu^{3.49(113)}$. The structural information strongly implicated the anti-clockwise packing orientation because TM III and VII are properly orientated to support these interactions. The clockwise orientation places these residues beyond a range for meaningful functional interactions (Baldwin, 1993).

Another issue that has caused significant anxiety within the molecular modeling field has been that of helical tilt angles. The early projection studies of bacteriorhodopsin determined that the helices in bacteriorhodopsin were primarily perpendicular to the plane of the lipid (Henderson, 1990). This packing was assumed correct for the entire superfamily of GPCRs and many molecular models were generated which appeared to be consistent with experimental SAR data (Donnelly *et al.*, 1994; Trumpp-Kallmeyer *et al.*, 1992; Maloney-Huss and Lybrand, 1992, Cronet *et al.*, 1993). When investigators began to think about whether or not this packing was consistent with the transduction of a signal through G-protein, it was unclear because bacteriorhodopsin acts as an ion channel and not a GPCR (Pardo *et al.*, 1992). With the recent elucidation of the helical packing arrangement of rhodopsin (Schertler *et al.*, 1993; Schertler and Hargrave, 1995; Unger and Schertler, 1995; Unger *et al.*, 1997), it appeared that helical tilt angles are different between the photoreceptors. Rhodopsin appeared to have only four helices perpendicular to the plane of the membrane (TMS IV, V, VI, and VII). TMS I-III appear to be significantly tilted and projected as a single horseshoe shaped density. These differences result in changes in the packing arrangement of the ends of the helices when viewed from either the extracellular or the intracellular face (Baldwin, 1993). The extracellular face of the receptor appears to have a more open conformation whereas the intracellular face of the receptor is more tightly packed. The closely packed intracellular face of rhodopsin brings all the regions known to be important for G-protein activation in very close proximity. The packing within the interior portions of the photoreceptors only change slightly (Baldwin, 1993; Unger *et al.*, 1997).

An additional structural feature of a majority of GPCRs, and not bacteriorhodopsin, concerns a disulfide bridge present on the extracellular side of the receptors. This disulfide bridge is formed between a highly conserved cysteine residue on the extracellular side of TMS III and additional cysteine residue located within the second intracellular loop. The average length of the second intracellular loop is very short, consisting of as few as 12 amino acid residues. The conserved cysteine residue is usually located within five amino acids from the start of TMS V. Therefore, for this disulfide bridge to be maintained the extracellular portions of TMS III and V must be in close proximity. The rhodopsin packing arrangement places these helices at a distance close enough to maintain a disulfide bond. However, the packing arrangement of bacteriorhodopsin places TMS IV between the ends of TMS III and V reducing the possibility of a stable disulfide bond very significantly (Baldwin, 1993).

A substantial amount of mutagenic data based upon the cationic amine binding receptors also supports the packing arrangement of rhodopsin for a template for the superfamily of GPCRs as opposed to bacteriorhodopsin. In all cationic amine-binding receptors, the cationic amine binds to a conserved aspartate residue in TMS III. For adrenergic receptors binding NE and Epi, additional interactions are made with serine residues located in TMS V. Furthermore, for muscarinic receptors binding acetylcholine similar ancillary interactions are made with threonine residues in TMS V. Although the packing arrangement of bacteriorhodopsin is less favorable when attempting to accommodate short ligands within the known binding site, the rhodopsin helical packing arrangement offers proper alignment of these residues for ligand interactions (Baldwin, 1993).

The use of GPCR TM domain molecular models to rationalize structure-activity relationships (SARs) and molecular mechanisms of receptor activation, however, requires the identification of important receptor residues involved in defining the binding site. In addition, it requires the use of energy minimization techniques to dock the ligand within the TM segments of the GPCR (Nguyen and Case, 1985). As pointed out in recent reviews, however, there are significant differences between published models of specific GPCRs, such as the β_2AR (Strader *et al.*, 1994; Maloney-Huss and Lybrand, 1992; Trump-Kallmeyer *et al.*, 1992). Therefore, it is surprising that all published models are claimed to be consistent with ligand SARs. This apparent paradox can be resolved, however, by observing that many of these studies only employed energy minimization methods. It is clear that more sophisticated techniques of evaluating the validity of structural models must be employed if they are to be used in any predictive manner. Molecular dynamics (MD) simulations of ligand/receptor complexes represent an interesting approach to discriminating between various models (Ballesteros and Weinstein, 1995). It has been shown that in MD studies, more reasonable agonist/receptor complexes appear to possess greater molecular flexibility than those formed from antagonists (Zhang and Weinstein, 1993; Luo *et al.*, 1994; Strahs and Weinstein, 1997). This observation is consistent with the current hypothesis of G protein activation via conformational changes in the βAR (Gether *et al.*, 1995; Gether *et al.* 1997; Gether and Kobilka, 1998). Analysis of average inter-pharmacophore distances in ligands derived from MD simulations also appears to be a potential method for the rational computational

evaluation of receptor/ligand complexes, when combined with results from site-directed mutagenesis studies (Ballesteros and Weinstein, 1995).

β_2-Selective Agonists

Development of Short-Acting Agonists

A major advance in the treatment of asthma occurred in the late 1960's and early 1970's with the introduction of β_2AR selective agonists. This selectivity increased the margin of safety of these drugs by reducing cardiovascular and other adverse effects. Many of these advances have been gained using structure activity relationships that have allowed for the rational design of subtype selective agonists.

Structure activity relationships have formed the cornerstone of medicinal chemistry and the chemistry of α and βAR agonists have been explored for the past four decades. However, the work to understand the structure-activity relationships of agonist that interact with the βAR has primarily focused on the stereochemical requirements of the principle class of agonists, the phenethylamines. The phenethylamines include the natural endogenous agonists, NE and Epi, as well as the synthetic agonists, ISO, salbutamol, and salmeterol. The general conclusions regarding the activity of these β-agonists are that the stereochemistry of the β-hydroxyl-substituted phenethylamines can affect the activity of the agonist greater than 100-fold (Ruffolo, 1991). Additionally, aromatic hydroxyl substitutions are also critically important for the β-agonist activity of the phenethylamines. The 3,4-dihydroxy phenethyl derivatives have the greatest agonist activity. The removal of either the *para*- or *meta*-substituted hydroxyl moieties results in a reduction in both the affinity and efficacy of the ligands for both the β_1 and the β_2ARs

(Waldeck *et al.*, 1986). Interestingly, replacement of the *meta*-hydroxyl group with substituents capable of forming a hydrogen bond results in compounds with potent βAR agonist activity. Specifically, the *meta*-hydroxymethyl derivative of (±)-*t*-butylnoradrenaline, a weakly selective, full β_2-agonist, is the β-agonist, (±)-salbutamol. Salbutamol is a representative of a family of salgenin-based agonists. This agonist has a relative activity greater than 600 times higher for the β_2AR as compared to the β_1AR. This difference in relative activity is primarily due to salbutamol having a much greater efficacy when stimulating the β_2AR (Ruffolo, 1993; Coleman and Farmer, 1971; Coleman and Nials, 1986). However, if the *para*-hydroxyl functional group is exchanged for the hydroxymethyl moiety, then the agonist activity at the β_2AR is lost (Brittain *et al.*, 1970). The effects of N-substitution of phenethylamines have been highly documented (Triggle, 1981) and are most important in developing agonist selectivity for the βARs over the αARs. Additionally with both the catechol and salgenin based agonists, more potent β_2AR agonists have been obtained simply by replacing the N substituents with larger non-polar substituents (Brittain *et al.*, 1970; Brittain *et al.*, 1976; Ruffolo *et al.*, 1993).

The use of structure activity relationships in medicinal chemistry has led to an understanding of the functional characteristics of agonists that are selective for the β_2AR. Potent adrenergic agonists have a β-hydroxyl moiety in the R-stereoisomer and have important catechol hydroxyl moieties similar to the naturally occurring agonists, Epi and NE. Epi is more selective for the β_2AR as compared to NE due primarily to the methyl-substituted nitrogen. Enhanced β_2AR selectivity can be achieved with the catecholamine

pharmacophore by increasing the hydrophobicity of the N-substituents. Additionally, the β_2AR selectivity of the N-substituted catecholamines can be greatly enhanced by substitution of the *meta*-hydroxyl group on the phenyl ring with a *meta*-hydroxymethyl moiety to achieve a salgenin pharmacophore. This structural combination was employed to achieve the highly selective β_2AR agonist, salbutamol, which has achieved great success in the clinic for the treatment of asthma.

Background of Long-Acting or Slow-Dissociating β_2AR Ligands

Multiple daily dosing regimes are required for salbutamol due to the agonist having a relatively short half-life. This fact makes compliance and protection from bronchospasm throughout the night difficult. The duration of action of a drug can be prolonged by altering its mode of administration (slow release), developing a slow converting prodrug, or reducing its rate of metabolism and/or excretion. Another approach is to develop drugs that have a slow dissociation rate from its target receptor. In general, almost all βAR antagonists and agonists employed clinically or used in structure-function investigations exhibit relatively rapid dissociation kinetics. Sustained receptor occupancy therefore depends upon maintaining concentrations of the free drug near the receptor, the *in vivo* duration of action being altered by changing the distribution or metabolism of the compound to sustain efficacious plasma levels. However, several βAR antagonists have been introduced which have slow dissociation rates from the receptor producing insurmountable effects. These include FM-24 (Lucas *et al.*, 1979), ICI 147,798 (Keith *et al.*, 1989), nebivolol (Pauwels *et al.*, 1988) and tertatolol (De Blasi *et al.*, 1986). Slow dissociating compounds have also been reported for other GPCRs including

histamine (Brittain *et al.*, 1985), 5-HT (Bond *et al.*, 1989), and thromboxane A_2 (Olgletree *et al.*, 1993).

Major advantages of slow dissociating drugs include achievement of relatively stable receptor occupancy after initial dosage, and a duration of action that is insensitive to variations in the free drug concentration. An additional benefit of developing such drugs is a reduction in the incidence of side effects due to lower systemic concentrations of the free agonist. Several of these antagonists have been shown to produce sustained *in vivo* responses that are independent of plasma drug levels (Keith *et al.*, 1989; De Blasi *et al.*, 1986; Le Fur *et al.*, 1980). Of more interest in rational drug discovery, however, has been the observation that several of these antagonists exhibit slow dissociation kinetics at a particular receptor subtype (Keith *et al.*, 1989; Pauwels *et al.*, 1988). If this difference in kinetic behavior arises from structural differences in the receptor/antagonist complexes, then characterization of its molecular basis might be exploited to obtain new drugs that are highly subtype selective. To date, the mechanism underlying antagonist slow dissociation does not appear to have been delineated.

Several agonists have been shown to exhibit a sub-type selective slow dissociation from the β_2AR. The mechanism of action of at least one compound has been extensively studied and has been shown to be a receptor-based phenomenon (Green *et al.*, 1996). In general, however, much less is known about the effects of slow dissociating βAR agonists. Almost all studies of agonist/β_2AR interaction, and its consequences for receptor activation, have been carried out using ligands exhibiting rapid dissociation kinetics. Salmeterol is a derivative of salbutamol in which a ten-carbon chain with an

oxygen linker in the sixth position and a vicinal phenyl ring was added to the salgenin pharmacophore. Recently, salmeterol has been introduced into clinical practice for the treatment of asthma. Salmeterol is similar to other β_2-AR agonists in that it is able to cause brochodilatation of tracheal smooth muscle. However, salmeterol is distinct in that it has a slow onset of action, a very prolonged duration of action and its action is resistant to washout (Jack, 1991; Johnson *et al.*, 1993; Ball *et al.*, 1991; Nials *et al.*, 1993; Bradshaw *et al.*, 1987). Although the action of salmeterol can be blocked by antagonists, relaxation of the smooth muscle reappears without a second application of the drug following a washout period in which the antagonist is removed (Johnson *et al.*, 1993; Ball *et al.*, 1991; Nials *et al.*, 1993). This unusual pharmacology of salmeterol lead to the hypothesis that two binding sites for salmeterol existed (Jack, 1991; Johnson *et al.*, 1993). The first site was thought to be the standard agonist-binding pocket that would specifically bind the salgenin moiety to achieve its agonist activity. The second was a theoretical exosite that would bind the long hydrophobic tail at such a high affinity as to tether the salgenin moiety in the vicinity of the receptor (Clark *et al.*, 1996). This model suggested that the agonist activity reasserted itself after antagonist blockade by flapping the salgenin moiety in and out of the agonist-binding site. The extended duration of action of salmeterol was not observed for the β_1- or β_3-ARs (Kalish *et al.*, 1993). The absence of the long duration at these receptor subtype may be explained by the differences in affinity at the β_1- and β_3-ARs, 10,000- and 900-fold more selective, respectively, compared to the β_2-AR (Johnson and Coleman, 1995). Additionally it was thought that the mechanism of action involved tight binding of part of the molecule to an

exo-receptor site, or by a slow release of accumulated drug from the plasma membrane (Johnson, 1995; Anderson *et al.*, 1994). However, recent experiments employing mutant β_1 and β_2ARs in which non-conserved amino acids of the fourth TM segment are exchanged for the cognate sequence have confirmed that the mechanism of salmeterol's extended duration of action is receptor-mediated. Additionally, these chimeric β_1/β_2ARs have identified a discrete region of the β_2AR that confers the persistent receptor activation following washout. All of these changes occurred without affecting the affinity of salmeterol for the receptor (Green *et al.*, 1996). This data provides significant evidence for the hypothesis that a specific exosite exists for salmeterol. This specific exosite is located within the intracellular 1/3 of TM IV of the β_2AR itself and not within the membrane or other membrane constituent, and not associated with the agonist-binding pocket (Green *et al.*, 1996).

Although the structural determinates of salmeterol that give rise to slow-dissociation from the β_2AR seems to have been identified, the molecular mechanism for the general phenomenon of slow-dissociating ligands has not been elucidated. To identify a common mechanism underlying slow-dissociation, the mechanism of action of structurally distinct ligands that display an enhanced duration of action is required.

Carbostyril-Based Long-Acting β_2AR Selective Agonists

In 1976, a series of βAR agonists based upon 8-hydroxycarbostyril were synthesized (Yoshizaki *et al.*, 1976; Yoshizaki *et al.*, 1977). Several of these compounds were very potent at stimulating βAR responses and had good chemical stability. In 1987, Milecki *et al.* published a report describing additional 8-hydroxycarbostyril derivatives in

which various side-chain phenyl substitutions were synthesized. One of these derivatives, carbo-Br, contained a reactive bromoacetamide moiety in the *para*- position of the side chain phenyl ring (carbostyril **5**, Figure 1-2). Carbo-Br, which after apparent covalent binding to the receptor, acted as an irreversible β-agonist. Carbo-amine, however, was another derivative that contained a *para*-amino substituent on the side-chain phenyl ring (carbostyril **2**, Figure 1-2). Carbo-amine, carbostyril **2**, was a potent β-agonist that produced antagonist-insensitive activation of adenylyl cyclase activity in rat reticulocyte membranes (Standifer *et al.*, 1989). Furthermore, pretreatment of reticulocyte membranes with carbostyril **2** reduced the β-AR content that was not reversed by extensive membrane washing, but recovery of the receptors occurred by incubation of the membranes at elevated temperatures. This data indicated that carbostyril **2** bound to the β-AR in a non-covalent manner and slowly dissociated from it. It is well known that β-agonists tightly bind to the β-AR during ternary complex formation consisting of the agonist, receptor and G_s. In the presence of guanine nucleotide, the complex is destabilized and agonist affinity is greatly reduced mainly by an increase in dissociation rate (Williams and Lefkowitz, 1977; De Lean *et al.*, 1980; Heidenreich *et al.*, 1980). However, the tight binding of carbostyril **2** to the β-AR appeared to be independent of ternary complex formation because both functional and binding assays were performed in the presence of a guanine nucleotide to maintain the receptor in the agonist low affinity state (Standifer *et al.*, 1989). More recently TA 2005 (carbostyril **9**, Figure 1-2), a 8-hydroxycarbostyril derivative related to carbostyril **2**, was shown by radioligand binding assays and in isolated tissue studies to also tightly bind to the β-AR (Voss *et al.*, 1992).

Although a large number of studies have been reported to develop highly potent and selective β-agonists, including several using the 8-hydroxycarbostyril (Yoshizaki *et al.*, 1976; Yoshizaki *et al.*, 1977; Hieble, 1991; Ruffolo *et al.*, 1995), the chemical structures of 8-hydroxycarbostyri-based β-agonists that contribute to high affinity binding and/or slow dissociation have not been defined.

Carbostyril **1** (Figure 1-2) is an additional full β-agonist with a potency for stimulating AC in the low nanomolar range. To further characterize the insurmountable activity of carbostyril **1**, Dr. Baker's Laboratory has investigated the effects of carbostyril **1** and ISO on cAMP accumulation in intact hamster DDT_1 MF-2 (DDT) cells, which contain only a β_2AR system. Both agonists (1 μM of either) produced a time-dependent increase in cAMP accumulation (Figure 1-3, Panel A). When an excess of propranolol (20 μM) was added after 4 min of incubation with ISO, the cAMP content decreased indicating a rapid blockade of the receptors (replacement of ISO with the antagonist). In contrast, the addition of propranolol had no subsequent affect on the carbostyril stimulated cAMP accumulation. This result shows that carbostyril **1** produces an antagonist-insensitive response in these cells, consistent with carbostyril **1** being a slow dissociating β-agonist. Although a phosphodiesterase (PDE) inhibitor (rolipram, 50 μM) was used in these experiments to retard cAMP metabolism, the concentration used did not completely inhibit PDE. The decrease in cAMP levels is therefore likely arising from residual metabolism. In similar experiments using rat C62B (C6) cells, which contain mainly β_1ARs, both ISO (1 μM) and carbostyril **1** (1 μM) were found to stimulate cAMP accumulation. In sharp contrast to our observations for DDT cells, however, when

propranolol (20 μM) was added after 4 min of incubation, the cAMP content decreased at the same rate for both agonists, indicating rapid receptor blockade by the antagonist. Therefore, carbostyril **1** dissociates from the β_1AR with similar kinetics to ISO (Figure 1-3, Panel B).

The affect of pre-incubating C6 and DDT cell membranes with ISO (1 μM) or carbostyril **1** (0.5 μM) on the receptor content (B_{max}) was determined by Scatchard (1949) analysis of (-)-[^{125}I]-iodocyanopindolol ([^{125}I]-CYP) binding (Figure 1-4). After a 15 min incubation (in the presence of Gpp(NH)p) with either agonist followed by washing (5 cycles at 4°C), there was no change in the receptor content in the C6 membranes. In contrast, pre-incubation with **1** induced a 46% reduction of the receptor content in DDT membranes while ISO had no affect. There was no change in the K_D value of [^{125}I]-CYP binding in any of the experimental groups (data not shown). These data confirm that carbostyril **1** tightly binds to the β_2AR but dissociates rapidly from the β_1AR. We note that the fraction of receptors with bound carbostyril **1** may have been greater than the observed 46 % given that some agonist may have dissociated during the 60 min incubation at 36 ^{0}C with [^{125}I]-CYP.

To eliminate the variable that differences in membrane constituents in DDT and C6 cells contribute to slow dissociation of carbostyril **1**, murine L cells were transfected with cDNAs encoding each of the human β_1AR and β_2ARs (see Chapter 2 for procedure). The affect of ISO (1 μM) and carbostyril **1** (1 μM) on cAMP accumulation was determined after a 12 min incubation with L cells expressing each of the receptor subtypes (Figure 1-5). Although these time course experiments were conducted in a

similar manner to our experiments employing DDT or C6 cells (Figure 1-3), here we only show the cAMP level at the 12 min time point. Both ISO (1 μM) and carbostyril **1** (1 μM) produced a time-dependent increase in the cAMP content of the transfected cells. Specific [^{125}I]-CYP binding or βAR agonist stimulated cAMP accumulation was not detected in untransfected cells. The addition of propranolol (20 μM) after 4 min of incubation with ISO resulted in a decrease in the cAMP content in cells expressing either receptor subtype. However, propranolol (20 μM) added after 4 min of incubation with carbostyril **1** resulted in a decrease in the cAMP level in cells expressing the β_1AR but had no affect in cells expressing the β_2AR. We then determined the affect of carbostyril **1** on the receptor content (B_{max}) after pre-incubation of the transfected cell membranes with the ligand. Pretreatment with carbostyril (0.5 μM) for 15 min, followed by washing, reduced β_2AR content by 49 % but had no affect on β_1AR levels (Figure 1-6). The K_D values for [^{125}I]-CYP binding were not significantly different in the various treatment groups (data not shown), again suggesting carbostyril **1** showed slow dissociation properties at the human β_2AR but not the human β_1AR and that differences observed in the DDT and C6 cells were not due to differences in membrane composition.

Several experimental results are consistent with the hypothesis that non-covalent, tight binding of the carbostyril derivatives at the β_2AR is a direct consequence of their intermolecular interaction with the receptor alone. The tight binding is not arising from some other affect such as tight agonist binding in the agonist-receptor-G_s ternary complex. The first result was that insurmountable binding does not occur at the β_1AR although it can form a ternary complex, identical to the β_2AR. Secondly, in competition

assays, guanine nucleotides that destabilize the ternary complex and increase agonist dissociation have little affect on the potency of these compounds to inhibit radioligand binding. Additionally, the presence of guanine nucleotides does not prevent insurmountable binding in membrane preparations, or intact cells, where intracellular GTP is present and the ternary complex is probably transient. Heat treatment of membrane preparations has little affect on the receptor but destroys the ability of G_s to form a ternary complex. Carbostyril **2** still binds to receptors uncoupled by thermal methods in an insurmountable manner (data not shown). The cAMP accumulation in response to stimulation by carbostyril **1** and **2** is antagonist insensitive and selective for only the β_2AR.

We therefore conclude that the slow dissociation properties selective for the β_2AR of some carbostyril-derived β-agonists are not due to their sequestration in lipophilic, non-receptor sites. However, the mechanism does appear to be receptor-based in a similar fashion to that proposed for salmeterol (Green *et al.*, 1996). Interestingly, while retaining a significant potency, all of these compounds dissociate rapidly from the β_1AR. Our initial observations have been confirmed with a closely related derivative (Voss *et al.*, 1992). Definition of the structural basis of this subtype-dependent kinetic behavior would therefore represent important new information that will facilitate the rational discovery of novel, highly selective β-ligands.

Specific Aims of the Current Project

Currently, there is an increasing number of slow dissociating receptor ligands which have potential therapeutic utility, and which should have significant

pharmacological advantages in terms of duration of action and a reduction in side effects. We have discovered a series of carbostyril-based β-agonists, which offer a unique opportunity to delineate the nature of the intermolecular interactions that confer slow dissociation properties on these ligands at the β_2AR. The approach taken in this dissertation uses an interactive, multidisciplinary experimental approach (pharmacology, hmolecular biology and molecular modeling). In addition, molecular biological and computational tools for investigating the role of specific amino acids in ligand binding and dissociation kinetics are well developed for the β_2AR. Elucidation of a structural model for the complex between the β_2AR and slow dissociating carbostyril β-agonists may provide information that will enhance our ability to discover novel classes of potent, highly subtype selective, β-agonists. The specific aims of this study were to identify the residues important for the binding of the carbostyril pharmacophore using site directed mutagenesis, to identify the important functional groups present on carbostyril **1** which are responsible for the receptor subtype selective slow dissociation from the β_2AR using SAR analysis, to identify the region of the β_2AR which confers slow dissociation of carbostyril **1** from the β_2AR using chimeric β_2/β_1ARs, and to attempt to integrate this information in a comprehensive computer-based model of the complex between carbostryil **1** and the β_2AR. The proposed experiments have provided new information on the molecular interactions that give rise to slow dissociation of carbostyril **1** from the β_2AR.

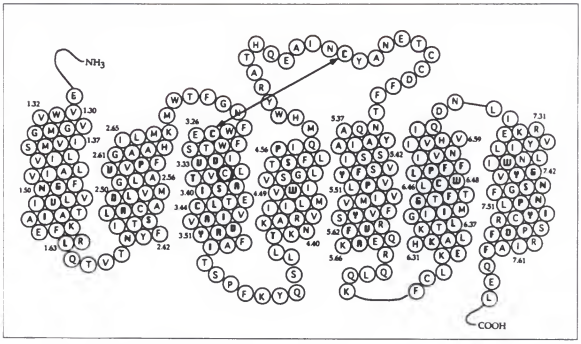
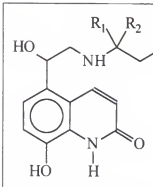


Figure 1-1. **Helical net representation of the human β_2AR.** TM domains with all intracellular and extracellular loop regions are indicated. The N-terminal, C-terminal, third extracellular loop and third intracellular loop regions are abbreviated due to the size. The highly conserved amino acid residues are shown in bold. A disulfide bond formed by cysteines in TM domain II and the second extracellular loop is indicated by the arrow. The TM domain boundaries are shown as reported by Donnelly *et al.* (1994). Figure was adapted from Ballesteros and Weinstein (1995).



1: $R_1 = R_2 = Me$, $R_3 = C_6H_5$
2: $R_1 = R_2 = Me$, $R_3 = C_6H_4NH_2$
3: $R_1 = R_2 = Me$, $R_3 = C_6H_4NHCOCH_2Br$

Figure 1-2. **The carbostyril ligands employed in studies of the insurmountable agonist activity selective for the β_2AR.** Carbostyril **1**, **2**, and **3** have been historically regarded as carbo-parent, carbo-amine and carbo-Br, respectively (Milecki *et al.*, 1987; Standifer *et al.*, 1989).

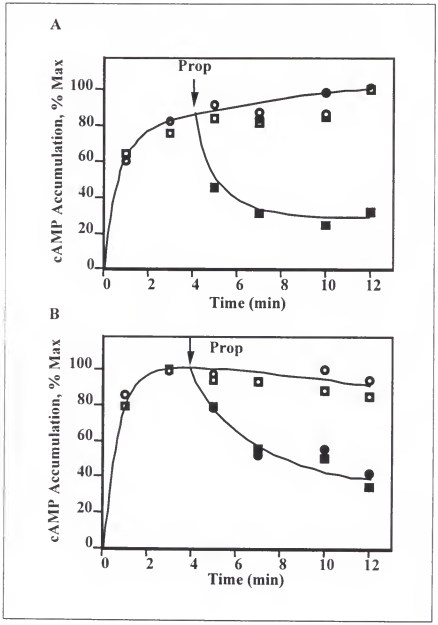


Figure 1-3. **Effect of carbostyril 1 on cAMP accumulation in DDT and C6 cells.** DDT (Panel A) or C6 (Panel B) cells were incubated with ISO (1 μM, open squares) or carbostyril **1** (1 μM, open circles) in the presence of 50 μM rolipram. After 4 min of incubation, propranolol (20 μM, arrows) was added to parallel groups (filled symbols). At the times indicated, the cAMP content was determined. Maximum cAMP formed in the DDT and C6 cells was 440 and 400 pmol/mg protein, respectively, at 12 min (n=3).

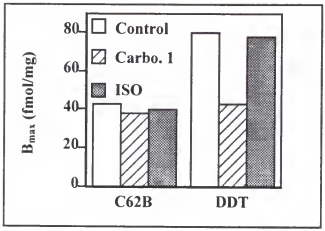


Figure 1-4. **Effect of carbostyril 1 on βAR in C6 and DDT cell membranes.** Cell membranes were incubated with ISO (1 μM) or carbostyril **1** (1 μM) for 15 min at 36 ^{O}C. Membranes were then washed 5 times and the B_{max} determined from Scatchard analysis of [^{125}I]-CYP binding.

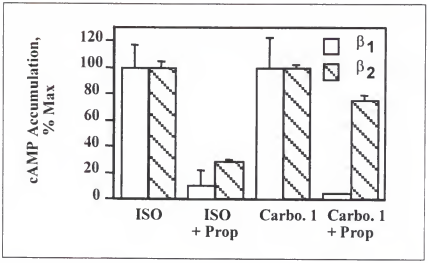


Figure 1-5. **Effect of carbostyril 1 on the cAMP accumulation in transfected mouse L cells.** Cells were incubated with 50 μM rolipram and ISO (1 μM) or carbostyril **1** (1 μM) at 36 ^{o}C. After 4 min of incubation, propranolol (20 μM) was added to parallel assays. The cAMP accumulated was determined at 3 min intervals and the 12 min time point is shown. The maximal β_1AR stimulation was 91 and 105 pmol/mg/3 min for ISO and carbostyril **1**, respectively. The maximal β_2AR stimulation was 141 and 163 pmol/mg/3 min for ISO and carbostyril **1**, respectively (n=3).

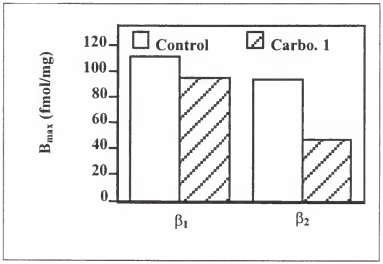


Figure 1-6. **Effect of carbostyril 1 on the βAR in transfected mouse L cell membranes.** Cell membranes of murine L cells expressing either the β_1AR or the β_2AR protein were incubated in the presence of 100 μM Gpp(NH)p. and 0.5 μM carbostyril **1** for 15 min at 36 ^{O}C. The membranes were then extensively washed and the B_{max} was determined by Scatchard analysis of [^{125}I]-CYP binding.

CHAPTER 2
EXPERIMENTAL PROCEDURES

Materials

DNA modifying enzymes were purchased from either Promega (Madison, WI) or New England Biolabs (Beverly, MA). Alpha-[^{33}P]-dATP (2000 Ci/mmol), (-)-[^{125}I]-iodocyanopindolol (2200 Ci/mmol) and Na[^{125}I] (2200 Ci/mmol) were purchased from DuPont/NEN (Wilmington, DE). All oligonucleotide primers were synthesized using an Applied Biosystems 380B DNA synthesizer in the DNA Synthesis Core Facility of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. The Wizard MiniPrep kit, employed in all preparations of plasmid DNA, and the Altered Sites II Mutagenesis kit were from Promega (Madison, WI). DNA sequencing kit (Sequenase, v. 2.0) was from U.S. Biochemical Corp. (Cleveland, OH). TRIzol Reagent, the Superscript II Preamplification System, Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), Geneticin (G-418), penicillin, streptomycin, Opti-MEM I and LipofectAMINE were purchased from Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Mediatech, Inc. (Herndon, VA). The mammalian expression vector pCLDN-10B was a generous gift from Smith Kline Beecham (King of Prussia, PA). Murine L and DDT_1 MF-2 (DDT) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). CGP 12177 was from Research Biochemicals International (Natick, MA). All other chemicals, including adenosine 3',5'-cyclic monophosphate (cAMP), 5'-

guanylylimidodiphosphate (Gpp(NH)p), (-)-isoproterenol, (+)-isoproterenol, dobutamine, hydroxyapatite, and ScAMP-TME, were purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). Glass fiber filters were from Schleicher & Schuell (Keene, NH).

Methods

Receptor Nomenclature

A common numbering scheme was developed by Ballesteros and Weinstein (1995) to relate site-defined properties to sequence data from many different classes of GPCRs. The numbering scheme contains identifiers concerning the relative position of each amino acid within a helix, the particular amino acid present at that position, and the actual amino acid number of that particular GPCR being discussed. Each amino acid identifier begins with the TM domain number and is followed by the position relative to a reference residue in that TM domain. The reference residue is the most conserved amino acid in that particular TM domain and is given the arbitrary position number 50. Following the particular identifier, the actual amino acid number is included in parenthesis. Examples of this numbering scheme can be illustrated by the conserved tryptophan at position 158 in TM domain IV of the β_2AR. The nomenclature for this amino acid would appear $W^{4.50\,(158)}$. A serine residue in the same helix, seven residues from this particular tryptophan would have an identifier, $S^{4.57\,(165)}$. Table 2-1 lists all of the conserved reference amino acids for each TM domain with the actual amino acid numbers for the β_2AR. Mutations can also be noted with this nomenclature simply by

placing the new amino acid in that position after the WT identifier. For example, a tryptophan to phenylalanine mutation in TM IV would appear as $W^{4.50\,(158)}F$.

Table 2-1.
Generalized numbering scheme for GPCR sequences[a].

TM domain	100 % conserved in GPCRs	AA Identifier	AA No. in β_2AR	AA identifier in β_2AR
I	Asn	N 1.50	51	N 1.50 (51)
II	Asp	D 2.50	79	D 2.50 (79)
III	Arg	R 3.50	131	R 3.50 (131)
IV	Trp	W 4.50	158	W 4.50 (158)
V	Pro	P 5.50	211	P 5.50 (211)
VI	Pro	P 6.50	288	P 6.50 (288)
VII	Pro	P 7.50	323	P 7.50 (323)

[a] Adapted from Ballesteros and Weinstein, 1995.

Standard nomenclature was used to describe the TM domain composition of chimeric receptor constructs obtained from Dr. Thomas Frielle at the University of Pennsylvania Medical Center, Hershey, PA (Frielle *et al.*, 1988). The nomenclature can be summarized in one example. The β_2(I-IV)/β_1(V-VII) AR chimera contains the TM domains I-IV of the β_2AR amino acid sequence and TM domains V-VII of the β_1AR.

An additional nomenclature was employed to describe the amino acid substitutions present in the βAR chimeric constructs obtained from Dr. Stuart Green at the University of Cincinnati Medical Center, Cincinnati, OH. All of these constructs are described by the dominant sequence characteristic followed by the amino acid sequence that was substituted from the other subtype. For example, β_2S(149-173) is the β_2AR with amino acids 149-173 exchanged for the cognate sequence present in the β_1AR (Green *et al.*, 1996).

Mutagenesis

DNA sequences encoding WT β_2AR in the pSP65 vector were subcloned into the vector, pALTER-1. $S^{4.53(161)}A$, $S^{4.57(165)}A$, $S^{5.43(204)}A$, $S^{5.46(207)}A$ and $D^{3.32(113)}E$ β_2AR mutant sequences were generated by site-specific mutagenesis using the oligonucleotides 5'-GTG TGG ATT GTG GCT GGC CTT ACC TCC-3', 5'-TCA GGC CTT ACC GCA TTC TTG CCC ATT-3', 5'- GCC ATT GCC TCT GCG ATC GTG TCC -3', 5'- CAT TGC CTC TTC GAT CGT GGC CTT CTA CGT TC -3' and 5'- GTT TTG GAC TTC GAT CGA ACT GCT GTG CGT C-3', respectively. The mutagenesis was performed as described in the Altered Sites II mutagenesis kit from Promega. EcoRI fragments containing the mutant open reading frames were excised from pALTER-1, blunt-ended and subcloned into the BamHI/HindIII blunt-ended site of the mammalian cell expression vector, pCLDN-10B. Similar procedures were employed to obtain the pCLDN-10B construct containing the DNA sequence encoding the WT β_1 and β_2AR.

Plasmids (pSP65) containing chimeric β_1/β_2AR constructs (Frielle *et al.*, 1988) were obtained from Dr. Thomas Frielle at the Pennsylvania State University, Hershey, PA. Each open-reading frame was excised from pSP65 and subcloned into the multiple cloning site of pcDNA3 (Invitrogen, Carlsbad, CA) for eukaryotic cell expression.

βAR TM IV mutant receptor constructs obtained from Dr. Stuart Green at the University of Cincinnati Medical Center, Cincinnati, OH in the pBC12BI expression vector were generated as previously reported (Green *et al.*, 1996) and were used unmodified.

Cell Culture and Transfections

DDT cells were grown as monolayers on poly-*L*-lysine treated 150 mm plastic tissue culture dishes. The dishes were prepared by adding 10 ml of sterile water containing 0.01 mg / ml of poly-*L*-lysine and after 1 hr at room temperature they were rinsed with 2 x 5 ml of water. Murine L cells were grown as monolayers on plastic tissue culture dishes. DDT and murine L cells were grown in DMEM supplemented with 10 % FBS, 50 U / ml penicillin, and 50 μg / ml streptomycin (DDT cells: 2.5 μg / ml amphotericin B) in a humidified atmosphere of 5 % CO_2 / air at 37 ^{o}C. Cells were detached weekly for subculturing by incubation in HBSS (without divalent cations) containing 0.25 % trypsin and 1 mM EDTA. WT and mutant β_2AR pCLDN-10B plasmid constructs, and chimeric β_2AR constructs contained in pcDNA3 were transfected into murine L cells using plasmid DNA (1 μg) and the LipofectAMINE reagent (10 μg), using the manufacturers procedure. The βAR TM IV mutant constructs contained in the expression vector pBC12BI were co-transfected with the vector pSV_2neo (10:1 ratio) which confers G-418 resistance. Clonal cells resistant to 1 mg / ml G418 were isolated and assayed for [^{125}I]-CYP binding, or agonist induced stimulation of intracellular cAMP.

Membrane Preparation and Pretreatments

One-day preconfluent cells (DDT or murine L cells) were washed twice with 10 ml HBSS, scraped free of the dish in 4 ml of 50 mM Tris-HCl buffer at pH 7.4 at 4 ^{o}C (homogenization buffer). After homogenization, using a Tekmar homogenizer at setting 20 for 10 s, the resulting suspension was diluted to 35 ml in homogenization buffer and centrifuged at 27,000 x *g* for 10 min. The membrane pellet was resuspended in 4 ml of

homogenization buffer by vortexing and centrifuged as before. The final pellet was resuspended in 50 mM Tris-HCl at pH 7.4 containing 5 mM $MgCl_2$ (binding buffer) for assays. The protein content in the membrane preparation was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

In pretreatment experiments, DDT cell membranes (1 mg / ml protein) were incubated in 50 mM Tris-HCl at pH 7.4 containing 100 μM Gpp(NH)p and with or without 1 μM of the β-agonists for 4 min at 37 ^{O}C. At the end of the incubation, the suspensions were diluted to 35 ml with ice-cold buffer and centrifuged as before. These wash cycles of resuspension and centrifugation were repeated a total of six times. The final pellets were resuspended in 0.5 ml of binding buffer for assays.

Saturation Radioligand binding assays

The expression of βARs in DDT cells or in transfected murine L cells was assayed by specific [^{125}I]-CYP binding (Strader *et al.*, 1988b). Briefly, membrane protein (5-25 μg) was incubated in 0.25 ml of binding buffer containing [^{125}I]-CYP (3-100 pM) and 100 μM Gpp(NH)p (pre-treated membranes only) in the presence or absence of 3 μM alprenolol for 60 min at 36 ^{O}C. At the end of the incubation, each sample was then diluted with ice-cold binding buffer (3 ml) and the membrane-bound radioligand collected onto glass fiber filters using a Brandel cell harvester. After further washing of the filters with ice-cold binding buffer (6 ml), the bound radioactivity was determined by gamma spectroscopy. Specific binding to the βAR was calculated as the difference in total binding determined in the absence of (-)-alprenolol and the nonspecific binding determined in the presence of 1 mM (-)-alprenolol. Each assay was performed in triplicate and specific binding was usually 80-90 % of the total binding.

Competition Binding Experiments

In competition assays to determine the concentration of ligands that inhibited specific [^{125}I]-CYP binding by 50 % (IC_{50}), the assays were performed as above except the [^{125}I]-CYP concentration was maintained at 30 pM. Additionally, incubations were carried out in the presence 100 μM Gpp(NH)p in all experiments, except for those designed to detect high-affinity forms of the receptors.

Cellular Localization of Expressed Receptors

Experiments to determine the cellular locations of expressed β_2ARs were carried out using the [^{125}I]-CYP binding assay described with the following modifications. One-day preconfluent murine L cells were rinsed twice in HBSS, detached from the culture plates using a cell lifter, centrifuged at 500 x *g* for 5 min, and gently resuspended in HBSS. Cell viability was greater than 95 % during the assay as determined by trypan blue exclusion. Total and nonspecific [^{125}I]-CYP binding was determined using the procedure outlined above except that HBSS was employed as the incubation buffer. Receptor content was determined using 3 μM alprenolol and 3 μM CGP 12177, to block the total and cell surface receptors (Staehelin and Hertel, 1983), respectively. The number of β_2ARs expressed on the cell surface was calculated as the difference between the total and intracellular components.

Measurement of cAMP accumulation

DDT cells and murine L cells expressing either the WT or mutant human βAR were rinsed with HBSS (2 x 10 ml) and gently detached into HBSS (5 ml) using a cell lifter. The resulting suspension was centrifuged at 500 x *g* for 5 min. Aliquots of the cell

suspension (0.1 mg protein) were then preincubated in 0.4 ml HBSS for 5 min at 37 ^{O}C. At the end of this incubation, rolipram (50 μM) and the β-agonists indicated in the text were added and the incubation continued for the times indicated in the text. The resulting suspensions were placed in a boiling water bath for 5 min, cooled to room temperature and then centrifuged at 13,000 x *g* for 2 min. The cAMP content of the supernatant was determined by a modification of a radioimmunoassay (Harper and Brooker, 1975) described by Belardinelli *et al.* (1996). Briefly, an aliquot of the supernatant (10-50 μl) was diluted to 100 μl with HBSS containing 12,500 dpm of I-ScAMP-TME. After the addition of anti-cAMP antibody (50 μl of 1:2000 dilution with 0.1 % BSA in water), the samples were incubated at 4 ^{O}C for 18 hr before a 50 % (v/v) hydroxyapatite suspension was added to each tube (70 μl). These suspensions were incubated for 10 min at 4 ^{O}C. The antibody/radioligand complex adsorbed to the hydroxyapatite was retained on glass fiber filters by rapid filtration using a Brandel cell harvester. The filters were washed subsequently with ice-cold 10 mM Tris-HCl buffer at pH 7.0 (9 ml) prior to determination of bound radiolabel. Nonspecific binding of I-ScAMP was determined in parallel assays that contained 3 μM unlabeled cAMP, and was subtracted from total binding. The amount of cAMP was calculated from a standard curve of cAMP. All determinations were performed in triplicate. Data analysis was performed using the Prism software package (Graphpad, San Diego, CA).

Pretreatment of DDT Cells to Remove Receptor Reserve

Attached DDT cells were rinsed twice with 2 x 10 ml of HBSS and incubated for 20 min at 37 ^{O}C in 10 ml HBSS containing 1 nM 5[2-[[[1'-(4'isothiocyanatophenyl)thiocarbonyl]-amino]-2-methylpropyl]amino-2-

hydroxypropoxy]-3,4-dihydrocarbostyril (DCITC), a potent irreversible antagonist for the βAR (Deyrup *et al.*, 1998). At the end of the incubation, the cells were washed 15 times by the addition of ice-cold HBSS (10ml) followed 5 min later by aspiration to remove the buffer. After the final wash, the cells were detached prior to assay, as described.

Determination of Apparent Ligand Dissociation Rates

DDT cells and Murine L cells expressing exogenous receptor protein were cultured to one-day preconfluency and rinsed with a solution of HBSS (2 x 10 ml), prewarmed to 37 ^{O}C. The resulting cells were detached into HBSS (5mL) and the suspension centrifuged at 500 x *g* for 5 min. Aliquots of the cell suspension (0.1 mg protein) were prewarmed in HBSS (400 μL total volume) for 5 min at 36 ^{O}C and incubated in the presence of 1 μM agonist for 4 min. At the end of this incubation, propranolol was added to a final concentration of 20 μM (t = 0) and the incubation was continued. At varying times after the addition of propanolol, 0.1 ml of isobutylmethylxanthine (IBMX) was added to a final concentration of 0.5 mM and the tubes were placed in a boiling water bath for 5 min. The suspensions were then cooled to room temperature, centrifuged at 13,000 x g for 2 min. The cAMP content of the supernatants was determined as described above. All determinations were performed in triplicate. The apparent dissociation rate constant was determined by curve fitting the decay in cAMP accumulation to an exponential equation using the Prism software package (Graphpad, San Diego, CA).

Localization of Measured cAMP

In some experiments, the intracellular and extracellular cAMP content was determined. At the end of the incubation to stimulate cAMP accumulation as above,

IBMX was added to a final concentration of 0.5 mM and the cells were centrifuged at 13,000 x *g* for 2 min. The supernatant was saved for the determination of extracellular cAMP. The cell pellet was resuspended in 0.5 ml HBSS containing 0.5 mM IBMX, and the suspension placed in a boiling water bath for 5 min. After cooling to room temperature, the samples were centrifuged at 13,000 x *g* for 5 min and the cAMP content of the supernatant (intracellular cAMP) was determined.

Phosphodiesterase Activity

DDT cells were homogenized in 40 mM Tris-HCl buffer at pH 8.0 and the phosphodiesterase activity of the whole homogenate was determined as described by Belardinelli *et al.* (1995).

Data Analysis

The concentration of agonists that stimulated cAMP accumulation by 50 % of the maximal response (EC_{50}) and the concentration of ligands that inhibited [^{125}I]-CYP binding by 50 % (IC_{50}) were determined from nonlinear regression analysis using the Prism program (GraphPad Software Inc., San Diego, CA). The value of K_I for the displacement of [^{125}I]-CYP binding by each agonist was calculated using the Cheng and Prusoff (1973) transformation of the IC_{50} values. The K_D and maximal binding values (B_{max}) for [^{125}I]-CYP were determined form nonlinear regression analysis of Rosenthal (1967) plots (Prism Program). The occupancy-response relationship was fitted to a monophasic exponential association equation using nonlinear regression (Prism Program) and the receptor reserve for the maximal response defined as 90 % of the maximum was calculated from the standard curve. The apparent k_{off} for the agonists was determined by fitting the rate of cAMP decline date to a monoexponential decay equation using

nonlinear regression analysis (Prism Program). Statistical analysis of the data was performed using the student's t-test and differences were considered significant if $p <$ 0.05.

Chemical Synthesis

Analytical Procedures

Melting points were determined on a Fisher Jones melting point apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a QE-300 MHz spectrometer 300 MHz, FT mode) using Me_4Si as an internal standard. ^{13}C NMR spectra were obtained using a QE (75 MHz, FT mode) spectrometer. High-resolution mass spectra were obtained using Finnigan MAT 95Q or Finningan MAT LCQ in ESI mode. Compounds were purified by preparative TLC (Analtech, 20 x 20 cm, 2000 microns) or column chromatography on silica gel (Merck 70-230 mesh) as specified.

Synthetic Procedures

(±)-8-Methoxy-5-[1-hydroxy-2-N-[2-benzyl-1,1-dimethylethyl]amino-ethyl]-carbostyril (7). The carbostyril derivative (±)-**1** (35.2 mg, 0.1 mmol), prepared according to published procedures (Milecki *et al.*, 1987), dimethyl sulfate (12.6 mg, 0.1 mmol) and K_2CO_3 (7.6 mg, 0.11mol) were dissolved in 5:1 acetone/H_2O (3 ml), and the mixture heated for 1 h. After TLC analysis showed 50 % conversion of starting material, the acetone was removed and the aqueous phase extracted with chloroform (2 x 3 ml). The organic phase was dried ($MgSO_4$), and the solvent removed under reduced pressure. The desired O-methylated carbostyril (±)-**7** (10 mg, 27 %) was purified using preparative TLC (eluant: 10% MeOH in CH2Cl2): ^{1}H (300 MHz, CD_3OD) δ 9.24-9.23 (1H, br. s),

8.09 (1H, d, J = 10.0 Hz), 7.33-7.16 (6H, m), 6.97 (1H, d, J = 12.0 Hz), 6.69 (1H, d, J = 9.5 Hz), 3.97 (3H, s), 2.96-2.92 (2H, m), 2.68-2.62 (2H, m), 1.26 (6H, s); MS (ESI) m/z 349 (MH+ - H2O), 217, 202; exact mass calcd. for $C_{22}H_{27}N_2O_3$ requires 367.2021, found 367.2018.

(±)-1-Methyl-8-benzyloxy-5-oxiranyl-(1H)-quinolin-2-one (**B**). A solution of (±)-8-benzyloxy-5-oxiranyl-(1H)-quinolin-2-one **A** (2.56 g, 8.8 mmol) dissolved in dry DMF (10 ml) was added drop-wise to a suspension of NaH (530 mg of a 60 % suspension in oil, 13.2 mmol) in a mixture of DMF (20 ml) and THF (10ml). After stirring at room temperature for 1 h, neat MeI (0.85 ml, 13.2 mmol) was added slowly via syringe. Stirring was continued until TLC showed complete consumption of starting material, and then the reaction mixture was filtered and the solvent removed under reduced pressure. The crude residue was suspended in water (20 ml) and extracted with CH_2Cl_2 (3 x 50 ml). The organic extracts were dried ($MgSO_4$) and the solvent removed to give the N-methylated epoxide **B** as a yellow oil (3 g) that was used in the next step without further purification: 1H (300 MHz, CDCl3) δ 8.05-8.04 (1H, m), 7.46-7.36 (7H, m), 6.75 (1H, d, J = 9.5 Hz), 5.34 (2H, s), 4.22-4.19 (1H, m), 3.91 (3H, s), 3.23-3.18 (1H, m), 2.60-2.70 (1H, m).

(±)-1-Methyl-8-benzyloxy-5-[1-hydroxy-2-N-[2-benzyl-1,1-dimethylethyl]amino-ethyl]-(1H)-quinolin-2-one (**9**). A portion (1 g) of the crude, N-methylated epoxide **B** was mixed with neat phenteramine (970 mg, 6.5 mmol) and the reaction mixture heated for 5 h. Upon cooling, CH_2Cl_2 (100 ml) was added and the excess phenteramine removed by extraction with 1 % aq. HCl. The organic layer was washed with water (50 ml) and dried ($MgSO_4$). Evaporation of the solvent under reduced pressure gave a yellow oil that was

subsequently purified by column chromatography on silica (eluant: CH_2Cl_2, then 98:2 CH_2Cl_2/MeOH and finally then 95:5 CH_2Cl_2/MeOH) to give the desired product **9** as a yellow foam (218 mg): ^1H (300 MHz, $CDCl_3$) δ 8.03 (1H, d, J = 10.0 Hz), 7.43-7.09 (12H, m), 6.68 (1H, d, J = 10.0 Hz), 5.25-5.21 (1H, m), 5.08 (2H, s), 3.86 (3H, s), 3.03 (1H, d, J = 3.0 Hz), 2.99 (1H, d, J = 3.0 Hz), 2.83-2.77 (2H, m), 1.12 (3H, s), 1.11 (3H, s); ^{13}C (75.4 MHz, $CDCl_3$) δ 163.08, 147.08, 137.31, 136.07, 134.24, 132.58, 130.34, 128.60, 128.26, 128.19, 127.97, 127.56, 126.36, 121.46, 120.70, 120.15, 115.27, 72.52, 68.77, 54.42, 49.12, 47.32, 36.03, 26.37, 26.28; HRMS (EI) exact mass calculated for $C_{29}H_{33}N_2O_3$ requires 457.2491, found 457.2493.

(±)-8-Hydroxy-5-[1-hydroxy-2-N-[2-benzyl-1,1-dimethylethyl]amino-ethyl]-carbostyril (**8**). The O-benzylated amino-alcohol **C** (217 mg, 0.48 mmol) was dissolved in MeOH (15 ml). After the consecutive addition of 10 % Pd/C (60 mg) and 1,4-cyclohexadiene (0.42 ml, 4.3 mmol), the resulting suspension was heated for 2 h, when TLC analysis indicated complete consumption of starting material. After filtration through celite, and removal of solvent under reduced pressure, column chromatography on silica gel (eluant: 90:10 CH_2Cl_2/MeOH) gave the desired carbostyril derivative **8** as a yellow crystalline material (153 mg, 88 %): ^1H (300 MHz, CD_3OD) δ 8.20 (1H, d, J = 10.0 Hz), 7.28 (1H, d, J = 8.0 Hz), 7.26-7.13 (5H, m), 7.02 (1H, d J = 8.0 Hz), 5.29 (1H, t, J = 6.0 Hz), 3.94 (3H, s), 2.99 (2H, d, J = 6.0 Hz), 2.83 (2H, s), 1.15 (6H, s); HRMS (EI) exact mass calculated for $C_{22}H_{27}N_2O_3$ (MH+) requires 367.2021, found 367.2021.

8-Hydroxyquinoline-N-oxide (**D**). To a solution of 29.4 g (0.02 mol) of 8-hydroxyquinoline in methylene chloride (150 ml) in a 1 l round-bottom flask immersed in an ice bath, and equipped with an addition funnel (250 ml), m-chloroperbenzoic acid 59.4

g (0.02 mol) suspended in methylene chloride (350 ml) was added slowly with stirring. The solution became yellow, then thickened with a bright yellow precipitate. The mixture was stirred overnight at 0 ^{O}C. The next day an additional 200 ml of solvent was added to dissolve some of the precipitated product which was extracted with a saturated solution of sodium bicarbonate (2 x 150 ml). The organic layer was washed with water (200 ml), dried ($MgSO_4$) and the solvent was removed under reduced pressure. Yield was quantitative, m.p. 136-138 ^{O}C (lit. m.p. 138-139 ^{O}C. Shrader *et al.*, 1988). ^{1}H NMR (DMSO): 7.02 (1H, d), 7.41 (1H, d), 7.50-7.60 (2H, m), 8.09 (1H, d), 8.54 (1H, d). ^{13}C NMR (DMSO): 114.29, 117.31, 121.95, 128.21, 130.38, 130.63, 130.80, 132.93, 132.93, 135.64.

8-Acetoxycarbostyril (**E**). A mixture of 8-hydroxyquinoline-N-oxide **D** (20 g) and acetic anhydride (120 ml) was heated in a 1 l round-bottom flask on a water bath (80-90 ^{O}C) for 5 hrs. The dark solution was poured onto ice and slowly basified with concentrated ammonium hydroxide. Cooling continued for an additional 30 minutes and the golden solid was filtered off, washed with cold water (50 ml) and air-dried. The yield was 20 g (78 %). Recrystallization from 96 % ethanol gave an analytical sample with a m.p. of 253-254 ^{O}C (lit. m.p. 252-254 ^{O}C, Pettit *et al.*, 1968).

8-Hydroxycarbostyril (**F**). A suspension of 8-acetoxycarbostyril **E** (20 g) in concentrated hydrochloric acid (200 ml) was heated on a water bath (80-90 ^{O}C) for 4 hrs. After cooling, the dark suspension was poured onto ice and the light beige precipitate was filtered off, washed with cold water (50 ml) and dried. The yield was 14.2 g (90 %). Recrystallization from aqueous ethanol resulted in shiny crystals with a m.p. of 301-304

^{O}C (lit. m.p. 297-299^{O}C, Pettit *et al.*, 1968). ^{1}H NMR (DMSO): 6.55 (1H, d), 7.02-7.08(2H, m), 7.10-7.18 (1H, m), 7.90 (1H, d).

2-Hydroxy-2-[8-hydroxy-2(1H)-quinolinon-5-yl]ethanoic acid (**G**). Glyoxylic acid (50 % w/w aqueous solution 8.80 ml, 0.0798 mol) was added dropwise to a solution of 8-hydroxycarbostyril (25.65 g, 0.1595 mol) and KOH (13.3 g, 0.239 mol) in water (150 ml) at room temperature. The mixture was stirred at room temperature overnight and then neutralized with 5 % HCl. The mixture was allowed to stand overnight and the solids were filtered off (unreacted 8-hydroxycarbostyril) (15.16 g, 59 %). The filtrate was acidified with 5 % HCl to pH>1 and left at room temperature overnight. The solids that slowly precipitated were collected to give crude acid (12.70 g, 34l%, based on 41 % conversion). ^{1}H NMR (DMSO): 5.42 (1H, s), 6.58 (1H, d), 7.05 (2H, q), 8.32 (1H, d), 10.59 (1H, broad signal). ^{13}C NMR (DMSO): 68.74, 114.14, 118.19, 122.01, 122.43, 127.99, 129.03, 138.29, 143.94, 161.44, 174.76.

2-[8-Benzyloxy-2(1H)-quinolinon-5-yl]-2-hydroxyethanoic acid (**H**). Crude hydroxyacid **G** (12.57 g, 53.49 mmol), potassium carbonate anhydrous (8.86 g, 64.2 mmol), and KI (100 mg) were placed in a 500 ml round-bottom flask equipped with a U-tube, an addition funnel and a condenser protected from moisture. The mixture was refluxed in MeOH (150 ml) for 1 hr to ensure maximum dissolution of the hydroxyacid. Subsequently, benzyl chloride (9.23 ml, 80.2 mmol) was added dropwise to the mixture and refluxing was continued overnight. Water was added to dissolve the solid salts and methanol was removed on a rotary evaporator. The aqueous residue was extracted with methylene chloride and the aqueous phase was acidified by the addition of 5 % HCl with stirring. The sticky solids hardened overnight and were filtered off to give a crude

product that was used without further purification. An analytical sample was recrystallized from acetonitrile followed by methanol. M.p.155-157 ^{O}C. ^{1}H NMR (DMSO): 5.30 (2H, s), 5.40 (1H, s), 6.58 (1H, d), 7.15 (2H, d), 7.25-7.42 (3H, m), 7.55 (2H, d), 8.32 (1H, d), 10.70 (broad signal). ^{13}C NMR (DMSO): 70.25, 71.10, 111.24, 118.01, 122.03, 122.23, 128.18, 128.32, 128.48, 128.64, 128.78, 129.93, 130.10, 136.96, 138.30, 144.26, 144.26, 161.51, 174.71.

Methyl 2-[8-benzyloxy-2(1H)-quinolinon-5-yl]-2-hydroxy-ethanoate (**I**). The crude hydroxyacid **H** was refluxed in MeOH (150 ml) containing a catalytic amount of concentrated sulfuric acid (0.5 ml) overnight. The solid was collected by filtration and washed with cold methanol giving 12.75 g, 70 % yield after drying. Mp. 210-213 ^{O}C. ^{1}H NMR (DMSO): 3.58 (3H, s), 5.30 (2H, s), 5.52 (1H, d), 6.25 (1H, d), 6.58 (1H, d), 7.15 (2H, q), 7.30-7.45 (3H, m), 7.58 (2H, m), 8.25 (1H,d), 10.75 (1H, broad signal). ^{13}C NMR (DMSO): 52.29, 70.40, 70.96, 112.26, 117.87, 122.05, 122.65, 128.21, 128.33, 128.76, 129.05, 130.09, 136.94, 137.72, 144.59, 161.27, 173.47.

8-Benzyloxy-5-(1,2-dihydroxyethyl)-2(1H)-quinolinone (**J**). The hydroxyester **I** (6.3 g, 18.6 mmol) was suspended in 100 ml of dry THF, and lithium borohydride (5-fold excess, 46.5 ml, 2 M solution in THF) was added dropwise from an addition funnel under nitrogen. Methanol (50 ml) was subsequently added at a rate to avoid excessive foaming. The resulting homogeneous solution was stirred at room temperature for 4 hrs. Hydrochloric acid (5 %) was then added dropwise and the organic solvents were removed by evaporation. The aqueous residue was extracted with chloroform, and the organic extract was washed with water and brine. After drying and evaporation the crude product was purified by column chromatography on silica gel with chloroform-methanol. The

yield was 4.45 g (77 %). M.p. 110-112 ^{O}C. Found: C 65.48, H 5.81, N 4.20. For $C_{18}H_{17}NO_4$ x H_2O required: C 65.64, H 5.81, N 4.25. ^{1}H NMR (DMSO): 4.95-5.10 (2H, m), 5.20 (2H, s), 5.65 (1H, d), 6.55 (1H, d), 7.20-7.50 (5H, m), 7.60 (2H, d), 8.30 (1H, d), 10.70 (1H, broad signal). ^{13}C NMR (DMSO): 67.07, 69.69, 70.81, 111.96, 117.22, 119.84, 121.45, 127.68, 127.74, 127.77, 128.25, 128.99, 132.17, 136.61, 137.30, 147.08, 160.99.

2-[8-Benzyloxy-2(1H)-quinolinon-5-yl]-2-hydroxyethyl methanesulfonate (**K**). To the solution of dihydroxy derivative **J** (2.9 g, 9.3 mmols) in dry pyridine (50 ml), methanesulfonyl chloride (1.10 ml, 14 mmols) was added dropwise at 4 ^{O}C. The solution was precooled in an ice-salt bath prior to addition. The reaction was monitored by TLC. After 1 hr the reaction mixture was poured onto ice and the crude product was extracted with chloroform (3 x 100 ml). The organic layer was washed with 5 % HCl (75 ml), water, and brine, then dried ($MgSO_4$) and the solvent removed giving a crude product. It was purified by column chromatography on silica gel using methylene chloride - methanol (95:5) to elute the product. The yield was 2.42 g (67 %). M.p. 167-168 ^{O}C. Found: C 58.69, H 4.95, N 3.58. For $C_{19}H_{19}NO_6S$ required: C 58.61, H 4.92, N 3.60. ^{1}H NMR ($CDCl_3$): 3.01 (3H, s), 4.27 (2H, d), 5.20 (2H, s), 5.40 (1H, t), 6.45 (1H, d), 6.95 (1H, d), 7.25 (1H), 7.30-7.42 (5H, m), 7.98 (1H, d), 9.55 (1H, broad signal). ^{13}C NMR ($CDCl_3$): 37.68, 68.54, 70.90, 73.34, 111.29, 117.37, 121.02, 127.85, 128.15, 128.59, 128.78, 135.43, 136.38, 144.43, 161.70.

8-Benzyloxy-5-oxiranyl-2(1H)-quinolinone (**L**). To a finely powdered suspension of **K** (4.2 g, 11 mmols) in t-butanol (400 ml), potassium t-butoxide (6.05 g, 54.3 mmols) was added at room temperature. After 2 hrs the mixture was poured onto

ice and extracted with chloroform (3 x 200 ml). The organic layer was washed with water and brine, dried ($MgSO_4$) and the solvent was removed yielding a crude product. Recrystallization from a mixture of benzene-isooctane (2:3) (1000 ml) gave white crystals (2.88 g, 89 %). M.p. 120-122 ^{O}C (lit. m.p. 114-16 ^{O}C). Found: C 73.47, H 5.17, 4.74. For $C_{18}H_{15}NO_3$ required: C 73.71, H 5.15, N 4.78. ^{1}H NMR ($CDCl_3$): 2.66 (1H, m), 3.10 (1H, m), 4.10 (1H, m), 5.05 (2H, s), 6.60 (1H, d), 6.92 (2H, q), 7.25-7.45 (5H, m), 7.95 (1H, d), 9.32 (1H, broad signal). ^{13}C NMR ($CDCl_3$): 49.79, 49.86, 71.01, 111.10, 118.35, 119.02, 122.89, 126.57, 127.87, 128.61, 128.78, 128.88, 135.42, 136.02, 144.26, 161.46.

2-{2-Hydroxy-2-[8-benzyloxy-2(1H)-quinolinonyl]-ethylamino}propane (**O**).

Epoxide **L** (1.10 g, 3.82 mmols) and 2-amino-2-methylpropane (2 ml, excess) were heated neat for 4 hrs. The solvent was then removed under vacuum, the residue was dissolved in methylene chloride (30 ml) and extracted with HCl solution (15 ml, 1 %). The organic layer was washed with water (15 ml), dried ($MgSO_4$) and the solvent removed. Some of the product precipitated out from water as hydrochloride during washing with 1 % hydrochloric acid, and the rest was obtained by purification of the organic extracts on silica gel column using methylene chloride/methanol with increasing gradient of methanol. The yield was 0.91 g (76 %). M.p. 223-225 ^{O}C. Found: C 64.65, H 6.56, N 7.11. For $C_{21}H_{24}N_2O_3$ x HCl required: C 64.87, H 6.46, N 7.20. ^{1}H NMR (MeOD): 1.02 (3H, t), 1.75-1.83 (2H, m), 3.02-3.08 (2H, t), 3.19-3.22 (2H, m), 5.28 (2H, s), 5.42-5.55 (1H, m), 6.65 (1H, d), 7.18 (1H, d), 7.25-7.38 (3H, m), 7.46 (2H, d), 8.42 (1H, d). ^{13}C NMR ($CDCl_3$+MeOD): 11.28, 20.47, 50.62, 67.15, 71.80, 113.37, 118.68,

122.02, 122.51, 128.80, 129.29, 129.65, 130.34, 130.93, 137.53, 138.63, 142.37, 143.10, 143.21, 146.04, 162.00. HRMS 353.1816 (M+H). $C_{21}H_{25}N_2O_3$ requires 353.1865.

2-{2-Hydroxy-2[8-hydroxy-2(1H)-quinolinonyl]-ethyl amino}propane (**11**). The protected aminoalcohol **O** (2.6 g, 5.4 mmols) was dissolved in methanol (30 ml) and ammonium formate (500 mg) followed by palladium on charcoal (10 %, 100 mg) added at once under nitrogen. The reaction was heated until no more starting material was detected. The mixture was then cooled to room temperature, filtered through celite and the filtrate concentrated under vacuum. Crude product was purified by recrystallization from ethanol-ethyl acetate. The yield was 1.06 g (75 %). M.p. 235 ^{O}C. ^{1}H NMR (DMSO): 0.91 (3H, t), 2.42-2.61 (2H, m), 3.65-3.80 (4H, m), 5.43-5.46 (1H, m), 6.55 (1H, d), 7.02 (1H, d), 7.16 (1H, d), 8.29 (1H, d). HRMS 263.1326 (M+H). $C_{14}H_{19}N_2O_3$ requires 263.1395.

2-{2-Hydroxy-2[8-benzyloxy-2(1H)-quinolinonyl]-ethylamino}-2-methylbutane (**P**). Prepared according to the procedure as for **O**. The yield was 76 %. Product was recrystallized from absolute ethanol. M.p. 210-212 ^{O}C. Found: C 72.96, H 7.92, N 7.25. For $C_{23}H_{28}N_3O_3$ required: C 72.60, H 7.42, N 7.36. ^{1}H NMR (DMSO): 0.90 (3H, t), 1.30 (6H, s), 1.65-1.75 (2H, m), 2.90-3.10 (2H, m), 3.40-3.60 (2H, m), 5.35 (2H, s), 5.55-5.65 (1H, d), 6.61 (1H,d), 7.21-7.40 (5H, m), 7.60 (2H, d), 8.45 (1H, d), 10.85 (1H, broad signal). ^{13}C NMR (DMSO): 8.33, 22.31, 22.40, 30.25, 48.56, 59.82, 65.64, 70.15, 112.56, 116.93, 119.89, 122.92, 128.20, 128.30, 128.75, 129.81, 130.85, 136.86, 137.02, 144.17, 161.35. HRMS 381.2188 (M+H). $C_{23}H_{28}N_3O_3$ requires 371.2178.

2-{2-Hydroxy-2[8-hydroxy-2(1H)-quinolinonyl]-ethylamino}-2-methylbutane (**12**). Prepared according to the procedure as for **11**. Crude product was purified by

preparative TLC, using methylene chloride/methanol/ammonium hydroxide (7:3:0.2). The yield was 68 %. M.p. 176 ^{O}C (decomposition). ^{1}H NMR ($CDCl_3$+MeOD): 1.01 (3H, t), 1.38 (6H, d), 1.20-1.35 (2H, m), 3.10-3.35 (4H, m), 5.52 (1H, d), 6.65 (1H, d), 7.05 (1H, d), 7.35 (1H, d), 8.45 (1H, d). ^{13}C NMR ($CDCl_3$+MeOD): 6.90, 21.52, 21.64, 23.67, 30.23, 32.65, 60.15, 65.92, 113.84, 117.55, 120.82, 128.19, 128.19, 137.38, 144.0, 162.59. HRMS 291.1679 (M+H). $C_{16}H_{23}N_2O_3$ requires 291.1709.

5-[2-(N-Phenethyl)-1-hydroxyethylamino]-8-benzyloxy-2(1H)-quinolinone (**N**). Prepared according to the procedure as for **O**. Crude material was purified by trituration with ethyl acetate. The yield was 47 %. Purification of a small amount (100 mg) by column chromatography in methylene chloride - methanol (5 %), followed by crystallization from methylene chloride yielded analytically pure sample. M.p. 100-103 ^{O}C. Found: C 75.10, H 6.53, N 6.71. For $C_{26}H_{26}N_2O_3$ required: C 75.34, H 6.32, N 6.76. ^{1}H NMR (MeOD): 2.78-2.98 (6H, m), 5.09 (2H, s), 5.18-5.20 (1H, dd), 6.55 (1H, d), 6.92 (1H d), 7.12-7.26 (6H, m), 7.33-7.39 (5H, m), 8.08 (1H, d).

5-[2-(N-Phenethyl)-1-hydroxyethylamino]-8-hydroxy-2(1H)-quinolinone (**6**). Prepared according to the procedure for **11**. The yield was 85 %. The product was purified by column chromatography on silica gel using 5-7 % methanol in methylene chloride as eluting system. M.p. 132-134 ^{O}C. ^{1}H NMR (MeOD): 2.80-2.98 (2H, m), 3.01-3.08 (2H, m), 3.31 (2H, M), 5.10-5.15 (1H, m), 6.80 (1H, m), 6.90-7.0 (1H, m), 7.05-7.17 (6H, m), 8.25-8.40 (1H, m). HRMS 325.1552 (M+H). $C_{19}H_{21}N_2O_3$ requires 325.1551.

5-{2-[2-(Phenylprop-2-yl)amino]-1-hydroxyethyl}-8-benzyloxy-2(1H)-quinolinone (**Q**). Heating epoxide **L** (1.0 g, 3 mmol) with 1,1-dimethylbenzylamine

(1.89 g, 14 mol) overnight gave a crude product (Balderman and Kalir, 1978). The mixture was dissolved in methylene chloride (50 ml) and extracted with 1 % HCl, then washed with water and dried with $MgSO_4$. The solvent was removed under reduced pressure. The crude material was purified by dissolution in ethyl acetate and trituration with diethyl ether. The yield was 0.8 g (63 %). M.p. 239 ^{O}C (decomposition). ^{1}H NMR (DMSO): 1.82 (6H, d), 2.75-2.90 (2H, m), 5.30 (2H, s), 5.32-5.36 (1H, m), 6.55 (1H, d), 7.10-7.50 (10H, m), 7.60-7.65 (2H, m), 7.92 (1H, d). HRMS 429.2188 (M+H). $C_{27}H_{29}N_2O_3$ requires 429.2178.

5-{2-[2-(Phenylprop-2-yl)amino]-1-hydroxyethyl}-8-hydroxy-2(1H)-quinolinone (**13**). Adduct **Q** (0.3 g, 0.7 mmols), was heated with ammonium formate (150 mg) and palladium on charcoal (10 %, 30 mg) in methanol for 3 hrs. The catalyst was then filtered off and the solvent removed under vacuum. The crude product was purified on preparative TLC using 15 % methanol in methylene chloride. The yield was 170 mg (71 %). M.p. 160-162^{O}C. ^{1}H NMR (DMSO): 1.8 (6H, d), 2.75-2.85 (2H m), 6.50 (1H, d), 6.95 (1H, d), 7.15 (1H, d), 7.38-7.50 (3H, m), 7.60-7.65 (2H, m), 7.9 (1H, d), 8.50 (1H, m). HRMS 339.1730 (M+H). $C_{20}H_{23}N_2O_3$ requires 339.1708.

5-[2-(2-Methyl-5-phenylpent-2-yl)amino-1-hydroxyethyl]-8-benzyloxy-2(1H)-quinolinone (**R**). Epoxide **L** (1 g, 3 mmol) was heated with 2-amino-2-methyl-5-phenylpentane (1.2 g, 7 mmol) in n-butanol (5 ml) overnight to yield a crude adduct (Mousseron *et al.*, 1959). The solvent was removed under vacuum, and the crude mixture was dissolved in methylene chloride (50 ml). The mixture was then extracted with 1% HCl (30 ml), washed with water and dried ($MgSO_4$). After solvent removal, the product was recrystallized from ethanol. The yield was 0.9 g (64 %). A second

recrystallization from 2-propanol gave analytically pure product. M.p. 224-225 ^{O}C. ^{1}H NMR (DMSO): 1.32 (6H, s), 1.68 (4H, m), 2.59 (2H, m), 2.90 (2H, m), 5.40 (2H, s), 5.60 (1H, m), 6.25 (1H, s), 6.63 (1H, d), 7.22-7.42 (9H, m), 7.63 (2H, d), 8.47 (1H, d), 9.80 (1H, broad signal), 10.8 (1H, s). ^{13}C NMR (DMSO): 26.63, 24.93, 35.21, 36.63, 48.03, 59.02, 65.14, 69.67, 112.14, 116.48, 119.46, 122.52, 125.78, 127.78, 127.90, 128.25, 128.35, 129.40, 130.37, 136.35, 136.60, 141.61, 143.74, 160.94. HRMS 471.2641 (M+H). $C_{30}H_{35}N_2O_3$ requires 471.2648.

5-[2-(2-Methyl-5-phenylpent-2-yl)amino-1-hydroxyethyl]-8-hydroxy-2(1H)-quinolinone (**14**). Adduct **R** (0.235 g, 0.5 mmol) was dissolved in methanol (15 ml) and ammonium formate (150 mg) and palladium on charcoal was added (70 mg). The suspension was heated under nitrogen for 3 hrs. The reaction mixture was filtered through celite and concentrated. Yield was 0.12 g (63 %). M.p. 122-124 ^{O}C. ^{1}H NMR (DMSO) 1.23 (6H, s), 1.62 (4H, s), 2.50-2.60 (2H, m), 2.82-2.98 (2H, m), 5.40 (1H, d), 6.0-7.0 (broad signal), 6.60 (1H, d), 7.10-7.30 (6H, m), 8.25 (1H, d), 8.50 (1H, s). ^{13}C NMR (DMSO) 23.16, 25.09, 35.41, 37.10, 48.50, 58.20, 65.75, 114.25, 116.86, 120.0, 121.92, 125.87, 128.36, 128.58, 128.65, 136.81, 141.79, 144.14, 161.05, 166.79. HRMS 381.2146 (M+H). $C_{23}H_{29}N_2O_3$ requires 381.2178.

5-[2-(2-Propylamino)-1-hydroxyethyl]-8-benzyloxy-2(1H)-quinolinone (**S**). Epoxide **L** (0.6 g, 2.08 mmol) was heated with isopropylamine (4 ml, excess) in methanol (20 ml) overnight to yield crude adduct. The solvent and the excess amine were removed under vacuum and the crude material was purified by column chromatography using silica gel and methylene chloride-methanol as eluent. The product was eluted with 15 %

methanol in methylene chloride solution. The yield was 0.48 g (65.5 %). M.p. 122-123 ^{O}C. HRMS 353.1895 (M+H). $C_{21}H_{25}N_2O_3$ requires 353.1865.

5-[2-(2-Propylamino)-1-hydroxyethyl]-8-hydroxy-2(1H)-quinolinone (**15**).

Adduct **S** (0.3 g, 0.8 mmol) was dissolved in methanol (20 ml), and ammonium formate (150 mg, excess) was added followed by palladium on charcoal (100 mg, 10 %) as a catalyst. The mixture was heated for 20 min and then the catalyst was filtered through celite and the filtrate was evaporated to dryness. The yield was 150 mg (71 %). M.p. 145-149 ^{O}C. ^{1}H NMR (MeOD): 1.34 (6H, s), 3.19 (2H, s), 3.40-3.44 (1H, m), 5.42 (2H, s), 6.55-6.57 (1H, m), 6.99-7.05 (1H, m), 7.23-7.25 (1H, m), 8.27-8.29 (1H, m), 8.53 (1H, s). ^{13}C NMR (MeOD): 19.0, 52.0, 67.36, 67.44, 115.40, 118.96, 122.16, 129.29, 129.35, 129.40, 138.66, 145.48, 163.85. HRMS 263.1391 (M+H). $C_{14}H_{19}N_2O_3$ requires 263.1395.

1-{[2-[(1-Phenyl-2-methylprop-2-yl)amino]-1-hydroxyethyl]-3,4-bis(benzyloxy)}-benzene (**U**). 1,2-Bis(benzyloxy)-4-oxiranylbenzene **T** (2.89 g, 9.4 mmol) was reacted with freshly prepared phenteramine (3 ml, excess) and the mixture was heated under nitrogen for 6 hrs. The reaction was monitored by TLC. When epoxide was no longer detected, the mixture was diluted with methylene chloride (50 ml) and extracted with HCl (1 %) to remove unreacted amine. The organic layer was washed with water, and dried ($MgSO_4$). After the solvent was removed, the crude material was purified on silica gel column chromatography using 2-5 % methanol in methylene chloride as eluent. The yield was 1.84 g (43%). M.p. 121-122 ^{O}C. ^{1}H NMR ($CDCl_3$): 1.03 (3H, s), 1.05 (3H, s), 2.56 (2H, s), 2.58-2.68 (2H, m), 2.82-2.87 (1H, q), 4.49-4.53 (1H, m), 5.14 (2H, s), 5.16 (2H, s), 5.42-7.73 (18H, m). ^{13}C NMR ($CDCl_3$): 27.62,

26.68, 49.94, 54.08, 57.90, 67.23, 71.55, 71.64, 119.94, 126.14, 127.69, 127.72, 127.82, 128.37, 130.59, 137.35, 138.32. HRMS (M+H) 482.2695. For $C_{32}H_{36}NO_3$ required 482.2660.

1-{[2-[(1-Phenyl-2-methylprop-2-yl)amino]-1-hydroxyethyl}-3,4-bis(hydroxy)}-benzene (**16**). Adduct **U** (100 mg, 0.2 mmol) was dissolved in 10 ml of methanol. Catalyst (10 % Pd/C, 80 mg) was added followed by 1,4-cyclohexadiene (0.2 ml, 2 mmol). This mixture was refluxed until the starting material was no longer detected by TLC. The suspension was filtered through celite and then concentrated yielding a yellow solid (33.8 mg, 56 %). M.p. 102-105 ^{O}C (decomposition). ^{1}H NMR (MeOD): 1.09 (6H, s), 2.75-2.95 (2H, m), 3.34-3.63 (2H, m), 4.51-4.60 (1H, m), 6.73-6.89 (5H, m), 7.13-7.29 (5H, m). HRMS (M+H) 302.1755. For $C_{18}H_{24}NO_3$ required 302.1756.

CHAPTER 3
FUNCTIONAL CHARACTERIZATION OF THE SERINE^{165} TO ALANINE HUMAN β_2-ADRENERGIC RECEPTOR MUTANT

Introduction

The human β_2-adrenergic receptor (β_2AR) has been extensively studied as a model system for G-protein coupled receptors (GCRs) (Strader *et al.*,1994; Kobilka, 1992) and has been shown to bind a variety of catecholamine ligands. The βARs are members of a superfamily of genes whose encoded proteins are characterized structurally by seven hydrophobic, α-helical segments that traverse the plasma membrane, and which are thought to fold in a similar manner to that observed in electron cryomicroscopy studies of rhodopsin (Schertler *et al.*, 1993; Baldwin, 1993; Donnelly *et al.*, 1994). To date, three genes encoding βARs have been identified in the genomes of mammalian species (Frielle *et al.*, 1987; Kobilka *et al.*, 1987; Emorine *et al.*, 1989), and in all cases, recombinant βARs expressed in various mammalian cells display identical pharmacological profiles to their cognate native expressed receptors. Given its medicinal importance, the topology of the β_2AR has been investigated using various biochemical, molecular biological and immunological approaches. Several regions of the β_2AR have been mutagenized and the effects of these mutations on the function of the receptor assessed.

The ability to work with recombinant β_2ARs in site-directed mutagenesis experiments has provided detailed insights into the structural features important for catecholamine binding (Strader *et al.*, 1989a; Strader *et al.*, 1988; Strader *et al.*, 1989b; Wieland *et al.*, 1996; Dixon *et al.*, 1988). The results of these studies are consistent with the hypothesis that the ligand-binding pocket for biogenic amines is located within the TM regions of these GCRs. In particular, Asp$^{3.32(113)}$, located in putative TM domain III,

appears to interact with the side chain amino group of β-agonists through salt bridge formation, while $Ser^{5.43(204)}$ and $Ser^{5.46(207)}$ form hydrogen bonds to the *meta* and *para* phenolic groups of the catechol moiety, respectively. Additional mutagenesis experiments, in combination with molecular modeling studies, suggest that $Phe^{6.51(289)}$, $Trp^{6.48(286)}$ and $Phe^{6.52(290)}$ probably constitute a hydrophobic pocket in the β_2AR into which the aromatic moiety of the catecholamine ligand is bound (Strader *et al.*, 1994; Dixon *et al.*, 1988).

A specific hydrogen bond between the side chain of $Ser^{4.57(165)}$, in putative TM domain IV, and the stereoselective β-hydroxyl group of several agonists was predicted in many early models based on the bacteriorhodopsin TM structure (Strader *et al.*, 1994;Trumpp-Kallmeyer *et al.*, 1992). Initial attempts to express the $S^{4.57(165)}A$ hamster β_2AR mutant were unsuccessful (Strader *et al.*, 1989b), a result attributed to problems in obtaining correctly folded receptor protein. Recent mutagenesis experiments employing transient transfectants of the $S^{4.57(165)}A$ and $N^{6.55(293)}L$ human β_2AR mutants (Weiland *et al.*, 1996), however, suggest that this interaction is probably mediated by the side chain of $Asn^{6.55(293)}$. This observation is consistent with a recent model based on the packing coordinates of rhodopsin for the complex between NE and the TM domains of the human β_2AR (Donnelly *et al.*, 1994).

On the other hand, the experimental observation that $Ser^{4.57(165)}$ plays no apparent role in stereospecific agonist recognition raises new questions concerning its function in the β_2AR, given that this residue is conserved in the primary structures of all known adrenergic receptors. We now report a detailed pharmacological characterization of the $S^{4.57(165)}A$ mutant of the human β_2AR, as expressed in murine L cells. Competition experiments using radiolabeled antagonist and functional studies on cAMP accumulation provide confirmatory evidence against the presence of a specific hydrogen bond between the β-hydroxyl of ISO and the side chain of $Ser^{4.57(165)}$. More importantly, our results suggest that $Ser^{4.57(165)}$ functions to modulate the formation of the agonist/receptor/G_s

ternary complex, providing further support for the hypothesis that TM domain IV of the β_2AR is important in the molecular mechanism underlying the activation of guanine nucleotide binding protein by the receptor (Green *et al.*, 1993).

Methods

Confirmation of Transfected Receptor Genotype using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Individual murine L cell clones, expressing either WT or $S^{4.57(165)}$A human β_2ARs, were cultured as monolayers in growth media until they were one-day preconfluent. Total cellular RNA was isolated using the TRIzol Reagent, after washing each dish three times with HBSS. Single stranded cDNA was generated using oligo-dT primers and reverse transcriptase. A 643 bp segment of the cDNA produced from the mRNA encoding the β_2ARs was amplified using the following oligonucleotides as primers in the PCR: 5'-AAA CCA GAA TTG ACA TAG-3' and 5'-GCG AGT TTT GGA CAT CGA TTA ATG TGC TGT GCG T-3'. The amplification protocol (30 cycles) consisted of denaturation at 94 ^{o}C for 1 min, annealing at 50 ^{o}C for 2 min, and extension at 72 ^{o}C for 3 min. Each reaction (100 µl total volume) contained the cDNA mixture (5 µl), 2.5 U *Taq* DNA Polymerase (Promega), 1.5 mM $MgCl_2$, 100 µM dNTPs, and 0.1 µM of each primer in the thermobuffer. An aliquot of the PCR products were digested with *Bsa*MI and the products analyzed by gel electrophoresis through 1 % agarose (Figure 3-1).

Membrane Pretreatment with NEM

The level of ternary complex formation for the WT and $S^{4.57(165)}$A human β_2ARs was determined using *N*-ethylmaleimide (NEM), as previously described (Rigby and Wood, 1989). Briefly, membrane protein (1 mg) was incubated with 100 µM (-)-ISO in binding buffer (0.5 ml) in the presence or absence of 100 µM Gpp(NH)p for 2 min at 30 ^{o}C. Freshly prepared NEM was then added to a final concentration of 5 mM, and the suspensions incubated for a further 15 min. As a control, membranes were incubated

with NEM alone. Reactions were terminated by the addition of 20 mM cysteine (100 µl) and the samples were centrifuged at 13,000 x *g* for 2 min. The membranes were washed with ice-cold binding buffer (5 x 1 ml) by resuspension and centrifugation, as outlined above, and the final pellet was resuspended in 2 ml binding buffer for subsequent determinations of β_2AR content using [^{125}I]-CYP binding.

Results

DNAs encoding either the WT or S$^{4.57(165)}$A of the human β_2AR were expressed in murine L cells. In light of the reported problems in expressing the S$^{4.57(165)}$A hamster β_2AR mutant (Strader *et al.*, 1989b; Dixon *et al.*, 1988), several experimental approaches were employed to ensure that our constructs of the WT and S$^{4.57(165)}$A human β_2ARs gave functional expression in the murine L cell clones. We confirmed the presence of a unique *Bsa*MI restriction site in cDNA synthesized from mRNA encoding the S$^{4.57(165)}$A human β_2AR. Thus, single stranded cDNA was produced by reverse transcription of total cellular RNA isolated from each L cell clone, followed by the PCR amplification of a 643 bp region of the cDNA encoding the relevant fragment of the β_2AR (Figure 3-1). In cells expressing WT β_2ARs, the amplified fragment was not cleaved by *Bsa*MI (Figure 3-1, Lane 2). In contrast, *Bsa*MI treatment of the cognate fragment obtained from L cells transfected with the S$^{4.57(165)}$A human β_2AR construct yielded two fragments of the expected size (Figure 3-1, Lane 4).

The [^{125}I]-CYP binding parameters and cellular localization of the expressed WT and S$^{4.57(165)}$A β_2ARs were determined. Clonal cell lines were chosen so that receptor densities, derived by Scatchard analysis (1946), were approximately equal [WT β_2AR: 220 ± 40 fmol/mg protein; S$^{4.57(165)}$A β_2AR: 250 ± 40 fmol/mg protein]. More importantly, dissociation constants (K_D) for the radioligand at the two β_2ARs were similar, [WT β_2AR: K_D = 12.1 ± 0.7 pM; S$^{4.57(165)}$A β_2AR: K_D = 15 ± 2 pM]. These K_D values are consistent with those reported previously (Wieland *et al.*, 1996) and indicate

that the mutant receptor was folded correctly in the membrane. The fraction of cell surface β_2ARs was assessed by [^{125}I]-CYP binding to intact cells in experiments employing CGP 12177 and alprenolol as the cold competitors. CGP 12177 is relatively hydrophilic and therefore interacts only with β_2ARs located at the cell surface in an intact cell-binding assay (Staehelin and Hertel, 1983). In contrast, alprenolol penetrates the cell membrane and effectively binds to total cellular β_2ARs. Data from these experiments indicated that both the WT and S$^{4.57(165)}$A β_2ARs were present on the cell surface (Figure 3-2).

After identification of multiple L cell clones in which the levels of expressed WT and S$^{4.57(165)}$A β_2ARs were approximately equal as measured by [^{125}I]-CYP binding, we investigated the interaction of (-)-ISO with these receptors (Table 3-1) in both the presence and absence of Gpp(NH)p (Figure 3-3). In the absence of guanine nucleotide, inhibition of [^{125}I]-CYP binding at the WT β_2AR by (-)-ISO gave shallow curves that were best fitted to a two-site model (Hill slope = 0.59 ± 0.04). The high-affinity component represented 44 ± 6% of receptors with an affinity constant of 2.2 ± 0.6 nM, while the remainder possessed a lower affinity of 0.2 ± 0.1 μM. The competition curve was right shifted to a single low-affinity state in the presence of guanine nucleotide (Hill slope = 0.73 ± 0.04). Cell membranes containing mutant receptors displayed steep competition curves in both the presence and absence of guanine nucleotide and were only minimally rightward shifted by Gpp(NH)p. In the absence of Gpp(NH)p, (-)-ISO competition curves for the S$^{4.57(165)}$A β_2AR exhibited only a single low affinity component (Hill slope = 0.8 ± 0.1) of 0.4 ± 0.1 μM. In the presence of Gpp(NH)p, there was a 2.7-fold decrease in potency of (-)-ISO for the S$^{4.57(165)}$A β_2AR relative to that observed at the WT β_2AR (Table 3-1) with no change in the Hill slope (1.02 ± 0.09). Similar effects on potency, and the absence of high-affinity binding at the S$^{4.57(165)}$A β_2AR mutant, were observed for the stereoisomer (+)-ISO (Table 3-1). The reduction in high affinity agonist

binding in the mutant suggested that formation of the agonist high-affinity ternary complex by the $S^{4.57(165)}A$ β_2AR is impaired relative to the WT receptor.

Table 3-1.
Ligand binding parameters of WT and $S^{4.57(165)}A$ β_2ARs.

	WT β_2AR		$S^{4.57(165)}A$ β_2AR
	K_{low} (nM)	K_{high} (%) (nM)	K_{low} (nM)
(-)-ISO	200 ± 100	2.2 ± 0.6 (44 ± 6)	400 ± 100
(-)-ISO/Gpp(NH)p	220 ± 30	N/A	600 ± 100
(+)-ISO	3300 ± 900	60 ± 30 (48 ± 8)	10000 ± 3000
(+)-ISO/Gpp(NH)p	5500 ± 500	N/A	7700 ± 200

Each value is the mean ± S.E.M., n = 3 - 5 determinations.

An *N*-ethylmaleimide (NEM) and ISO pretreatment protocol was then used to further assess the ability of the two receptors to form the high affinity ternary complex. Formation of the ternary complex is thought to unmask sulfhydryl groups that are sensitive to chemical modification by NEM. Once modified, the stability of the receptor-agonist complex is greatly enhanced, preventing subsequent binding of radioligand and an apparent loss of receptor content (Rigby and Wood, 1989). Reductions in the observed receptor density after NEM/agonist pretreatment as measured by [^{125}I]-CYP binding are directly proportional to the fraction of ternary complex formed. It was observed (Figure 3-4) that a greater fraction of the WT β_2ARs expressed in murine L cells can form ternary complex when compared to the $S^{4.57(165)}A$ mutant [41.5 ± 0.2% (WT) *vs.* 20 ± 6% ($S^{4.57(165)}A$ mutant), $p < 0.05$]. This represents a 48% reduction in the amount of ternary complex formed. Addition of Gpp(NH)p prior to the addition of NEM (to abolish ternary complex formation) prevented the NEM/ISO-induced loss of [^{125}I]-CYP binding. Treatment of membranes with NEM alone caused no change in the number of WT or mutant β_2ARs (data not shown).

The consequence of reduced ternary complex formation found in membrane preparations isolated from cells expressing $S^{4.57(165)}A$ β_2ARs was evaluated functionally by determining the effects of β-agonists to stimulate cAMP accumulation in intact cells expressing approximately equal receptor densities of WT and $S^{4.57(165)}A$ β_2ARs. (-)-ISO

increased cAMP accumulation in cells expressing WT and $S^{4.57(165)}A$ β_2ARs in a concentration dependent manner (Figure 3-5 and Table 3-2).

Table 3-2.
EC_{50} values and maximal stimulation of agonist stimulated cAMP accumulation in cells expressing WT and $S^{4.57(165)}A$ β_2ARs.

	WT β_2AR		$S^{4.57(165)}A$ β_2AR	
	EC_{50} (nM)	Max %	EC_{50} (nM)	Max %
(-)-ISO	18 ± 3	100[a]	80 ± 20[b]	100[a]
(+)-ISO	370 ± 40	90 ± 10	2100 ± 400	91 ± 9
Dobutamine	270 ± 70	52 ± 5[c]	900 ± 100	15 ± 1[c]

[a]The maximal (-)-ISO stimulation of cAMP accumulation in cells expressing the WT and $S^{4.57(165)}A$ β_2AR was 217 ± 39 and 245 ± 23 pmol/mg protein/10 min, respectively.
[b]$p < 0.025$ vs. WT β_2AR
[c]$p < 0.005$ vs. respective (-)-ISO value
Each value is the mean ± S.E.M., n = 3 - 5 determinations.

Maximal responses to (-)-ISO were the same in cells expressing either receptor. However, the (-)-ISO concentration that produced half-maximal stimulation (EC_{50}) was 4.4-fold lower for the WT receptor compared to the $S^{4.57(165)}A$ β_2AR mutant. This difference in potency is consistent with the lower affinity found in radioligand competition assays using membrane preparations. Additionally, (+)-ISO was 21- and 26-fold less potent than the (-)-isomer for stimulating cAMP accumulation in cells with the WT and mutant receptors, respectively. The agonist effects of dobutamine, a known partial agonist for the β_2AR (Weber and Tuttle, 1977), were also investigated (Figure 3-4 and Table 3-2). The maximal stimulation of cAMP accumulation by dobutamine was 15% of the (-)-ISO maximum in cells with the $S^{4.57(165)}A$ receptor. This is a dramatic decrease from the 52% of the (-)-ISO maximum produced by dobutamine with cells expressing the WT receptor.

Discussion

Experiments involving the expression of site-specific mutants of mammalian βARs in various cell types have identified critical residues involved in β-agonist

recognition and binding (Strader *et al.*, 1989a; Strader *et al.*, 1988; Strader *et al.*, 1989b; Wieland *et al.*, 1996; Dixon *et al.*, 1988). However, the role of $Ser^{4.57(165)}$ in the β_2AR in the specific receptor/agonist interactions responsible for influencing the stereochemical preferences of the receptor were considerably less defined at the beginning of our investigations. In part, this was due to problems in expressing the hamster $S^{4.57(165)}A$ β_2AR mutant, an observation that was interpreted as implicating the conserved serine residue in either protein transport or folding (Strader *et al.*, 1989b; Dixon *et al.*, 1988). Early modeling studies were consistent with the side chain of $Ser^{4.57(165)}$ being involved in a hydrogen bond with the β-hydroxyl of catecholamine ligands (Strader *et al.*, 1994; Trumpp-Kallmeyer *et al.*, 1992). However, recent data suggests that $Asn^{6.55(293)}$ is the key residue in mediating ligand stereoselectivity (Donnelly *et al.*, 1994) and that $Ser^{4.57(165)}$ plays no role in ligand coordination. $Ser^{4.57(165)}$ is an example of a completely conserved residue among the adrenergic receptors in which the functional role remains unanswered. The goal of our work has been to determine the functional role of $Ser^{4.57(165)}$ in agonist activation of the β_2AR.

Restriction endonuclease analysis of a RT-PCR generated fragment of DNA synthesized from the total cellular RNA isolated from $S^{4.57(165)}A$ β_2AR DNA transfected cells confirmed our successful attempt to generate murine L cells expressing this mutant receptor. The presence of the unique restriction enzyme recognition site in this PCR product established the presence of the mutation within the expressed protein. Radiolabeled antagonist binding parameters and cellular localization studies indicated that the mutant receptor was not only folded correctly, but also accumulated on the cell surface. Therefore, $Ser^{4.57(165)}$ does not appear to be important for either the proper folding of the receptor or the accumulation of the receptor on the cell surface.

The decrease in the potency of (-)-ISO, in the presence of Gpp(NH)p, at the $S^{4.57(165)}A$ β_2AR was only 2.7-fold, in sharp contrast to the > 25-fold changes in potency of this agonist when $S^{5.43(204)}$ or $S^{5.46(207)}$ were replaced by alanine (Strader *et al.*, 1989b).

While our studies were in progress, a series of experiments utilizing cells transiently expressing mutant β_2ARs, showed not only a similar small decrease in (-)-ISO potency for the $S^{4.57(165)}A$ β_2AR as reported here, but also that replacement of $Asn^{6.55(293)}$ by a leucine dramatically reduced the stereoselectivity of the resulting β_2AR mutant (Wieland *et al.*, 1996). It seems likely that Asn^{293} participates in stereoselective interactions with the β-hydroxyl group of catecholamine ligands; therefore, $Ser^{4.57(165)}$ does not appear to coordinate with the ligand in this manner.

Although $Ser^{4.57(165)}$ does not mediate stereospecific ligand binding, this residue is conserved throughout the subfamily of adrenergic GCRs implying a functional role in the AR-subfamily. The relatively steep (-)-ISO competition binding isotherms observed for the $S^{4.57(165)}A$ β_2AR as indicated by a Hill-slope of 0.8 ± 0.1, coupled with the minimal effect of guanine nucleotide on agonist binding were consistent with this mutant receptor lacking a high-affinity βAR-agonist binding state (Figure 3-3), a conformation that likely mediates agonist-dependent activation of G-proteins. Despite the steep slopes and apparent absence of high affinity binding sites in competition assays, which indicates a total lack of ternary complex formation in cell membranes containing the $S^{4.57(165)}A$ receptors, we could detect ternary complexes, albeit at a reduced level, in the NEM/ISO pretreatment experiments. The level of ternary complex in membranes containing the WT receptor, estimated by chemical modification by NEM, was in good agreement with estimates from ISO competition of [^{125}I]-CYP binding in the absence of guanine nucleotide. Ternary complex estimates of the mutant receptor by the two methods were in less agreement. This difference may reflect a greater sensitivity to detect ternary complex formation in the NEM/ISO pretreatment protocol as compared to agonist competition of [^{125}I]-CYP binding. Nevertheless, both methods demonstrated the presence of significantly fewer ternary complexes in the membranes containing the mutant receptor when compared with the WT receptor. The simplest explanation for these observations is that the ability of the $S^{4.57(165)}A$ β_2AR to form a stable interaction

with G_s is impaired relative to that of the WT β_2AR. This hypothesis is also consistent with studies on a β_2AR variant present in the human population, in which $Thr^{4.56(164)}$ is substituted by isoleucine ($T^{4.56(164)}I$). Expression of the $T^{4.56(164)}I$ mutant β_2AR in CHW-1102 cells and subsequent measurement of ligand binding properties, demonstrated a 4.3-fold reduction in (-)-ISO potency, although the potency of β-agonists lacking a β-hydroxyl group was unchanged (Green *et al.*, 1993). More importantly, in agonist competition experiments conducted in the absence of guanine nucleotide, the $T^{4.56(164)}I$ mutant β_2AR also exhibited no detectable high affinity state. We interpret this to mean that TM helix IV plays a structural role in allowing formation of the agonist/β_2AR/G_S ternary complex.

The functional response of the $S^{4.57(165)}A$ receptor is consistent with decreased levels of ternary complex formation. As with the competition experiments, agonists displayed slightly higher EC_{50} values in elevating cAMP levels in cells containing mutant receptors. This decreased potency is also consistent with a decreased efficiency to form ternary complex in the mutant receptor. A significant decrease in the efficacy of the partial agonist, dobutamine, was detected at the $S^{4.57(165)}A$. These functional studies are again very consistent with results from both receptor-binding protocols in that there is a decreased level of ternary complex formation in the cell membranes containing mutant receptors. As has been shown previously, the efficacy of a partial agonist is directly related to its ability to form ternary complexes (Kent *et al.*, 1980). A strong correlation between the fraction of high affinity agonist binding sites and the intrinsic activity was shown for a series of βAR agonists. The significant decrease in efficacy of the partial agonist dobutamine in the cells expressing mutant receptors, therefore, could be explained by a hampered ability of the partial agonist to stabilize ternary complex formation in a mutant receptor already incapable of forming stable ternary complexes.

However, unlike the partial agonist responses, the efficacy of (-)-ISO was not diminished in the cells expressing mutant receptors, which contrasts with the results using

the $T^{4.56(164)}$I mutant receptor (Green *et al.*, 1993). This suggests that although ternary complex formation was reduced, enough complexes were formed to support maximal receptor stimulation of adenylyl cyclase activity.

The results of binding and functional assays using the $S^{4.57(165)}$A mutant β_2AR can also be explained in context of the extended ternary complex model (two-state model). It has been shown that ligand binding stabilize conformational changes involving TM III and TM VI that are proportional to the efficacy of the ligand tested (Gether *et al.*, 1995; Gether *et al.*, 1997). Additionally, molecular modeling of the β_2AR based on the rhodopsin projection map has suggested that $Cys^{3.44(125)}$ in TM III is bounded by the lipid bilayer and residues in TM IV. Also $Cys^{6.47(285)}$ is bounded at the interface of TM VII and the lipid bilayer (Gether *et al.*, 1997). In both cases it was suggested by the authors that receptor activation involves counterclockwise rotation of TM III and VI so that the cysteine residues would move from the non-polar lipid environment to the more polar interior of the protein (Gether *et al.*, 1997). These motions are stabilized by agonists to increase the proportion of receptors in the active conformation. Additionally, it appears that TM IV and VII are involved in the conformational changes responsible for the activation of the β_2AR. There are now two examples of mutations in TM IV in which polar residues were exchanged for non-polar residues produce receptors with impaired ability to activate G-protein (Green *et al.*, 1993; Nowicki *et al.*, 1997). As discussed, the $T^{4.56(164)}$I mutant β_2AR had a reduced ability to stimulate cAMP production in the presence of full agonists and was unable to form a high-affinity form of the receptor (Green *et al.*, 1993). The data presented in this chapter provide additional evidence for the role of polar residues in TM IV in stabilizing the active conformation of the receptor (Nowicki *et al.*, 1997). The $S^{4.57(165)}$A mutant β_2AR was impaired in its ability to form ternary complex and lacked high-affinity binding of agonists. Interestingly, this mutant β_2AR fully activated the adenylyl cyclase in the presence of full agonist as compared to the WT β_2AR.

However, the partial agonist dobutamine was impaired by an additional 61 % in its ability to stimulate a maximal accumulation of cAMP.

The two-state model is represented schematically in Figure 3-6 with the equations important for the derivation of basal activity, maximal activity, and the K_{app} of the ligand receptor interaction. This mathematical model was evoked to attempt to explain data obtained using the $S^{4.57(165)}A$ mutant β_2AR. The model was of particular use when attempting to explain the apparent paradox that the maximal response of ISO was not affected although the ability of the receptor to form stable receptor-agonist-G-protein ternary complex was diminished. Additionally, it was useful to explain the reduced efficacy observed for the partial agonist at the $S^{4.57(165)}A$ mutant β_2AR. Figure 3-7 shows the results of the theoretical mathematical modeling of the data in the context of the two-state model. As shown, the K_A and K_A^* values were constant for ISO and dobutamine at both the WT and $S^{4.57(165)}A$ mutant β_2AR. To accurately simulate the data, all that was required was to change the equilibrium constant for R and R*, L. A five-fold change in the equilibrium constant was all that was required to recreate the experimental data (Table 3-3).

Table 3-3.
Results of two-state model analysis.

	Full agonist L = 10 (WT)	Full agonist L = 50 (mutant)	Partial agonist L = 10 (WT)	Full agonist L = 50 (mutant)
K_{app}	11 nM	50 nM	550 nM	850 nM
Basal activity	0.09 %	0.02 %	0.09 %	0.02 %
Max activity	99.9 %	99.5 %	50 %	16.7 %

Full agonist: K_A = 1E-5 M, K_A^* = 1E-9 M
Partial agonist: K_A = 1E-6 M, K_A^* = 1E-7 M

Therefore, it appears that the only variable that has changed in the two state model is that the resting equilibrium between R and R* has changed so that less R* is stabilized by an interaction between TM IV and III. The 5-fold change in the equilibrium constant is equivalent to a 1.8 kJ/mole change which can be accounted for by the abolishment of the

formation of a single hydrogen bond in the receptor between the inactive R state and the active R* state.

In summary, the experiments described in this report all point to a decreased stability of the active conformation of the mutant $S^{4.57(165)}A$ β_2AR and therefore an overall reduction of agonist/receptor/G-protein complexes in the cells expressing these receptors compared to the WT β_2AR. $Ser^{4.57(165)}$ appears important for agonist-independent interactions within the receptor that allow interactions with the G-protein. This data further implicate TM helix IV as a critical determinant in stabilizing the active conformation of the receptor that allow agonist-dependent and agonist-independent interactions with the G-protein. In molecular dynamics simulations of the 5-HT_{2A} receptor structure, movements of TM helix IV occurred in response to receptor activation (Luo *et al.*, 1994). These movements affect the position of TM helix V leading to changes in the dynamics of the third intracellular loop region, the region that is directly responsible for the interaction between the receptor and G-proteins (Strader *et al.*, 1994; Kobilka, 1992; Dixon *et al.*, 1988). Changes in the dynamics of a fluorescently labeled β_2AR with ligands of different efficacies also correlate well with such a hypothesis (Gether *et al.*, 1995). For example, full agonists appear to induce the greatest changes in fluorescence, suggesting that the fluorophore moves into a hydrophilic pocket within the receptor core. With ligands of reduced efficacy, this movement was diminished in magnitude, supporting the concept of a coordinated rearrangement of the helices in response to agonist bindingthat transduce the signal from the ligand binding pocket to the third intracellular loop and G-protein. Residues located in transmembrane helix IV appear to be critical to this rearrangement and subsequent substitutions may therefore impair the ability of the receptor to change conformation in response to agonist binding.

Figure 3-1. **Confirmation of the $S^{4.57(165)}A$ mutation using RT-PCR and restriction endonuclease analysis.** The presence of a unique *Bsa*MI restriction site in cDNA synthesized from RNA encoding the $S^{4.57(165)}A$ human β_2AR was confirmed by RT-PCR analysis of total RNA prepared from one-day preconfluent murine L cells expressing either the WT or $S^{4.57(165)}A$ mutant β_2ARs. Shown is an ethidium bromide stained 1 % agarose gel containing PCR products reacted with and without *Bsa*MI. Lane 1: PCR product from cells expressing the WT β_2AR, Lane 2: PCR product from cells expressing the WT β_2AR reacted with *Bsa*MI, Lane 3: PCR product from cells expressing the $S^{4.57(165)}A$ mutant β_2AR, and Lane 4: PCR product from cells expressing the $S^{4.57(165)}A$ mutant β_2AR reacted with *Bsa*MI. In contrast, treatment of the cognate PCR product obtained from L cells expressing the $S^{4.57(165)}A$ mutant β_2AR with *Bsa*MI yielded two fragments of the expected size (468 and 175 bp). Numbers indicate the sizes of the migrated DNA standards (1 kbp ladder, Life Technologies, Inc.).

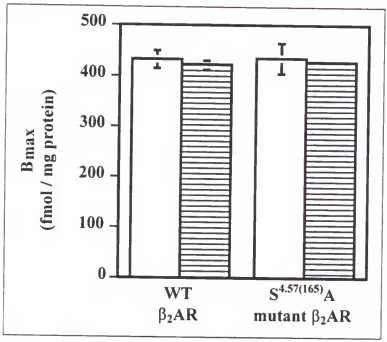


Figure 3-2. **Localization of the expression of the β_2AR with intact cell binding.** Whole murine L cells expressing either the WT or $S^{4.57(165)}$A mutant β_2AR were isolated. Receptor content was determined by [^{125}I]-CYP binding in HBSS buffer using either 3 μM alprenolol (total receptor content) or 3 μM CGP 12177 (intracellular content). The total receptor content is shown in the open bars. The cell surface fraction was determined by subtracting the intracellular fraction from the total receptor content and is shown in the hatched bars. Data shown are means ± S.E.M. of two independent experiments.

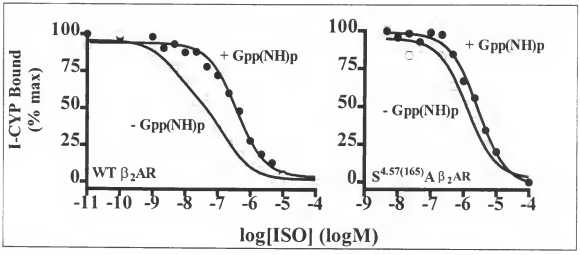


Figure 3-3. **Agonist competition with [^{125}I]-CYP for WT and S$^{4.57(165)}$A β_2ARs in the absence or presence of guanine nucleotide.** Membranes prepared from cells expressing either the WT or S$^{4.57(165)}$A β_2ARs were prepared and competition studies carried out as described. Panels A and B depict (-)-ISO competition binding curves with the WT and S$^{4.57(165)}$A β_2ARs, respectively, in the presence (filled circles) and absence (open circles) of Gpp(NH)p. The calculated Hill slopes for the competition of [^{125}I]-CYP binding by ISO at the WT and S$^{4.57(165)}$A β_2ARs in the absence and presence of guanine nucleotide were 0.59 ± 0.04, 0.73 ± 0.04, 0.8 ± 0.1 and 1.02 ± 0.09, respectively. The figure shows a representative analysis of multiple experiments performed with cell membranes containing WT (n = 6) and S$^{4.57(165)}$A (n = 5) β_2AR. Mean data are presented in Table 3-1.

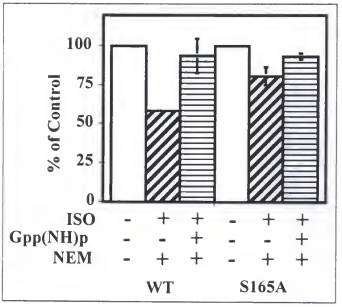


Figure 3-4. **Effect of pretreatment with (-)-ISO, Gpp(NH)p and/or NEM on [^{125}I]-CYP binding to membranes containing the WT and S$^{4.57(165)}$A β_2ARs.** Membranes (1 mg) from cells expressing either the WT or S$^{4.57(165)}$A β_2ARs were prepared, pretreated with 100 μM (-)-ISO and/or 100 μM Gpp(NH)p at 30 ^{o}C for 2 min, and treated for an additional 15 min with 5 mM NEM. The reaction with NEM was terminated by the addition of 20 mM cysteine. Membranes were extensively washed and assayed for [^{125}I]-CYP binding. Control receptor levels (100 %) were determined in the presence of buffer alone. Membranes treated with NEM alone showed no inhibition in radioligand binding. Data shown are means ± S.E.M. of two independent experiments.

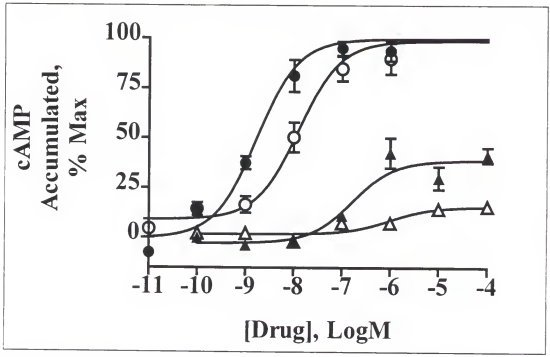
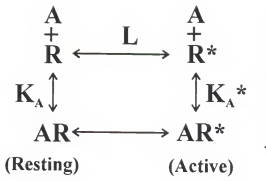


Figure 3-5. **Agonist-stimulated cAMP accumulation in cells expressing the WT and $S^{4.57(165)}A$ β_2ARs.** Representative experiment depicting agonist-dependent stimulation of cAMP accumulation in murine L cells expressing either the WT or $S^{4.57(165)}A$ β_2AR. Whole cells expressing either the WT or $S^{4.57(165)}A$ β_2ARs were prepared and the amount of accumulated cAMP in cells expressing either the WT (filled symbols) or $S^{4.57(165)}A$ (open symbols) β_2AR after exposure to various concentrations of either (-)-ISO (circles), or dobutamine (triangles) was determined. The maximal (-)-ISO stimulated cAMP accumulation was 200 ± 20 and 174 ± 13 pmol/mg protein/10 min for cells expressing WT and $S^{4.57(165)}A$ β_2AR, respectively. Data obtained with dobutamine are presented as the fraction of maximal cAMP accumulated in the presence of (-)-ISO for each cell type. Each point on the graph is the mean ± S.E.M. of quadruplicate determinations. Mean data is presented in Table 3-2.



$L = [R]/[R^*]$

$K_A = [R][A]/[AR]$

$K_A^* = [R^*][A]/[AR^*]$

$[R_{tot}] = [R] + [R^*] + [AR] + [AR^*]$

Therefore,

$$f_R^* = \frac{K_A^* + [A]}{K_A^*(1+L) + (1 + LK_A^*/K_A)[A]}$$

Additionally,

$$K_{app} = K_A^*(1 + L)/(1+LK_A^*/K_A)$$

$$\text{basal} = 1/(1 + L)$$

$$\text{max} = 1/(1 + LK_A^*/K_A)$$

Figure 3-6. **The two-state model of receptor activation.** The two-state model is shown schematically in which a receptor can exist in two states, R (resting) and R* (active). L describes the equilibrium constant in the absence of any ligand. K_A and K_A^* describe the binding constant for any ligand with affinity for the R and R* states of the receptor, respectively. Therefore the apparent binding affinity or potency and the efficacy are a function of L, K_A and K_A^*. For a complete explaination, refer to Chapter 1. Figure adapted from Leff, 1995.

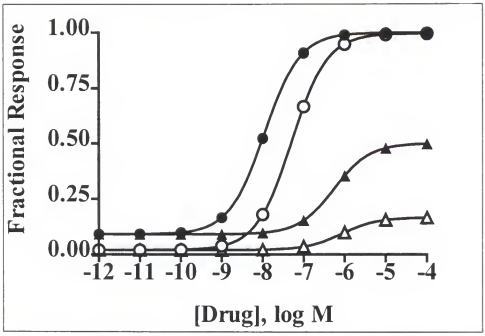


Figure 3-7. **Theoretical stimulation curves based on two-state mathematical model.** The stimulation curves were generated using the model and equations explained in Figure 3-6. The values of K_A and K_A^* for the full agonist (circles) were 1E-5 and 1E-9 M, respectively. The values of K_A and K_A^* for the partial agonist (triangles) were 1E-6 and 1E-7 M, respectively. The wild type receptor (closed symbols) and the mutant receptor (open symbols) had L values of 10 and 50, respectively. Theoretical analysis of curves presented in Table 3-3

CHAPTER 4
CHARACTERIZATION OF THE CARBOSTYRIL NUCLEUS BINDING SITE WITHIN THE β_2-ADRENERGIC RECEPTOR USING SITE DIRECTED MUTAGENESIS

Introduction

Our initial experiments will seek to define amino acids that interact with the carbostyril nucleus, and determine whether carbostyril **1** interacts with the β_2AR in a similar fashion as catecholamine ligands, e.g. ISO. Previous investigators have shown that substitution of $Asp^{3.32(113)}$, $Ser^{5.43(204)}$, and $Ser^{5.46(207)}$ with other amino acids yields mutant βARs with dramatically lower affinities for norepinephrine, epinephrine, and isoproterenol (Strader *et al.*, 1989b; Strader *et al.*, 1989c ;Strader *et al.*, 1988; Strader *et al.*, 1991). Replacement of $Asp^{3.32(113)}$ by asparagine yields a β_2AR mutant with an approximate 10,000-fold lower affinity for catecholamine agonists, while mutation of this residue to glutamic acid results in a β_2AR mutant with approximately 100-fold lower affinity for these agonists (Strader *et al.*, 1989c). Using site-directed β_2AR mutants, it has also been established that residues $Ser^{5.43(204)}$ and $Ser^{5.46(207)}$ in TM helix V are critical for catecholamine binding (Strader *et al.*, 1989b). However, $Ser^{4.53(161)}$ and $Ser^{4.57(165)}$ in TM helix IV do not appear to be involved in binding of ISO or catecholamines in general (Strader *et al.*, 1989a; Wieland *et al.*, 1996; Hehr *et al.*, 1997; Nowicki *et al.*, 1997). The systematic variation of residues identified in previous work on catecholamines was proposed to determine their role in carbostyril nucleus binding. In all of these studies,

stable mouse L cell transfectants expressing site-specific mutant β_2ARs were obtained and the effects of these mutations on agonist binding was determined. The affinity of the ligands was determined using competition radioligand binding experiments in the presence of [^{125}I]-CYP or by determining the potency of the compounds in adenylyl cyclase assays.

Ser$^{4.53(161)}$ has been implicated, in molecular modeling studies (Mitchell *et al.*, 1989), in forming a hydrogen bond interaction with the β-hydroxyl moiety of the catecholamine side chain. Only a two-fold decrease in the affinity of the hamster S$^{4.53(161)}$A β_2AR mutant for catecholamine agonists was observed (Strader *et al.*, 1989b). This change in affinity was interpreted to be too small if an important ligand-receptor hydrogen bond was being disrupted by the mutation. This particular mutation has been included as a negative control to insure that we can generate mutations proximal to the binding site within the β_2AR that have no effect on binding.

Additionally, site directed mutagenesis experiments, coupled with complementary ligand derivatization, are presented that show definitively that the carbostyril-based β-agonist **1** binds in the agonist-binding pocket as reported for catecholamines (Strader *et al.*, 1994; Weiland *et al.*, 1996). Finally, we show that the *N*-methylated derivative of this carbostyril-based ligand is an antagonist at the β_2AR, a surprising and unexpected observation in the light of previous SAR studies on carbostyril-based (Milecki *et al.*, 1987; Kikkawa *et al.*, 1997; Yoshizaki *et al.*, 1976) and catecholamine β-agonists (Ruffolo *et al.*, 1995).

Results

A series of single-point β_2AR mutants were prepared using oligonucleotide-directed site-specific mutagenesis so as to probe the interactions of carbostyril **1** with residues defining the agonist-binding site. Thus, $Asp^{3.32(113)}$ was replaced by glutamate, and $Ser^{5.43(204)}$ and $Ser^{5.46(207)}$ were substituted by alanine to give the $D^{3.32(113)}E$, $S^{5.43(204)}A$ and $S^{5.46(207)}A$ β_2AR mutants, respectively. The effects of these substitutions upon the binding affinity, potency and efficacy of carbostyril **1** and ISO at these β_2AR mutants, expressed in murine L cells, were assayed using radioligand binding and/or agonist-induced increases in intracellular cAMP (Table 4-1).

Table 4-1.
Equilibrium binding and adenylyl cyclase stimulation parameters for ISO and carbostyril 1, 7, and 8 for murine L cells expressing WT and mutant β_2ARs.

	ISO	Carbostyril **1**	Carbostyril **7**	Carbostyril **8**
WT β_2AR				
K_i (nM)	230 ± 30	3.2 ± 0.5	10 ± 2	10 ± 3
pEC50	8.77 ± 0. 03	9.3 ± 0. 2	7.1 ± 0.4	0
% Max	74.4 ± 0.7	70 ± 1	22 ± 2	0
$S^{5.43(204)}A$ β_2AR				
K_i (nM)	4900 ± 700	80 ± 30	130 ± 30	21 ± 3
pEC50	7.07 ± 0.02	7.8 ± 0.2	6.44 ± 0.08	0
% Max	61.2 ± 0.6	75 ± 4	20.8 ± 0.7	0
$S^{5.46(207)}A$ β_2AR				
K_i (nM)	3400 ± 500	23 ± 4	4.7 ± 1.0	13 ± 4
pEC50	7.3 ± 0.1	8.1 ± 0.1	8.7 ± 0.4	0
% Max	76 ± 3	43 ± 2	39 ± 2	0
$D^{3.32(113)}E$ β_2AR				
pEC50	5.1 ± 0.1	6.1 ± 0.1	NA	NA
$S^{4.53(161)}A$ β_2AR				
K_i (nM)	300 ± 100	2.4 ± 0.3	NA	NA

All results are presented as the mean ± S.E.M. of three to six experiments. NA indicates that it is not applicable to the investigation. % Max efficacy is reported as the percentage of the cAMP accumulated in the presence of 1 mM forskolin.

Antagonist-binding experiments demonstrated that the expressed WT β_2AR and the β_2AR mutants had similar K_Ds for [^{125}I]-CYP. The observed affinities of [^{125}I]-CYP for WT β_2AR and the $S^{4.53(161)}$A, $S^{5.43(204)}$A and $S^{5.46(207)}$A β_2AR mutants were 30 ± 3, 17 ± 2, 35 ± 3 and 35 ± 2 pM, respectively. [^{125}I]-CYP exhibited no affinity for the $D^{3.32(113)}$E β_2AR mutant, as reported previously (Strader *et al.*, 1989c). There was no significant change in the binding affinity of ISO for the $S^{4.53(161)}$A β_2AR mutant indicating that modifications can be made to the receptor without effecting the binding properties (Figure 4-3). However, changes were evident in the binding affinity of ISO at the $S^{5.43(204)}$A and $S^{5.46(207)}$A β_2AR mutants that are consistent with reported observations (Table 4-1 and Figure 4-3) (Strader *et al.*, 1989b). Additionally, the energetic contribution of the interaction between each serine and the catechol ring appeared to be similar. The affinity of carbostyril **1** for the $S^{5.43(204)}$A and $S^{5.46(207)}$A β_2AR mutants was decreased approximately 19- and 6-fold, respectively, when compared with the value observed at the WT β_2AR (Figure 4-3 and Table 4-1). The potencies of carbostyril **1** and ISO at the $D^{3.32(113)}$E β_2AR mutant were also determined. In this case, however, a dramatic reduction in the affinity of [^{125}I]-CYP precluded a direct assessment of ligand affinities using competition-binding experiments. Therefore measuring their ability to induce concentration-dependent increases in intracellular cAMP levels identified ligand affinities at this mutant receptor. Dramatic 8000- and 4000-fold reductions in potency were observed at the $D^{3.32(113)}$E β_2AR mutant for carbostyril **1** and ISO, respectively (Table 4-1).

It was established that carbostyril **1** and ISO formed similar interactions with the WT β_2AR. Therefore, Dr. Malgorzata Deyrup synthesized two carbostyril derivatives, **7**

and **8**, in which the 8-hydroxyl substituent was replaced by a 8-methoxy group and the secondary amide was N-methylated, respectively (Figure 4-1). The synthetic schemes used are described in Chapter 2 and Figure 4-2. The ability of these ligands to bind and stimulate WT β_2AR and the $S^{5.43(204)}$A and $S^{5.46(207)}$A β_2AR mutants were then determined (Figure 4-4, Table 4-1). Similar 3-fold reductions in binding affinity were observed for both **7** and **8** at the WT β_2AR as compared to carbostyril **1** (Table 4-1), although the abilities of these ligands to induce intracellular cAMP formation were unexpectedly different. While the O-methylated carbostyril derivative **7** was a partial agonist at the WT β_2AR, exhibiting a 7-fold decrease in potency with respect to carbostyril **1**, the N-methylated carbostyril **8** did not stimulate cAMP production (Figure 4-4D).

The effect of the $S^{5.46(207)}$A β_2AR mutation on the binding and activity of carbostyril derivative **8** was less dramatic, the affinity of this compound for this β_2AR mutant being identical to that at the WT β_2AR (Figure 4-4C). In addition, N-methylated carbostyril derivative **8** did not stimulate cAMP formation at this mutant receptor. More significantly, the affinity of the O-methylated carbostyril **7** was significantly higher than both carbostyril **1** and **8** at the $S^{5.46(207)}$A β_2AR mutant, being nearly identical to the affinity of carbostyril **1** at the WT β_2AR. Therefore, modification of carbostyril **1** by O-methylation had restored binding at the $S^{5.46(207)}$A β_2AR mutant. In addition, carbostyril **7** showed a dramatic increase in efficacy from about 21 % of the forskolin-stimulated maximum for the WT β_2AR to 39 % at the $S^{5.46(207)}$A β_2AR mutant (Figure 4-4F). This represents a 2-fold increase in the maximal amount of cAMP produced when compared with the maximum stimulated by carbostyril **7** at either the WT or the $S^{5.43(204)}$A mutant

β_2AR. The potency of carbostyril **7** at the $S^{5.46(207)}A$ β_2AR mutant was identical to that of carbostyril **1** at the WT β_2AR, and observation consistent with the results of the competition binding experiments.

In the case of the $S^{5.43(204)}A$ β_2AR mutant, dramatic decreases in affinity were seen with all three carbostyril-based ligands when compared with their affinity of the three ligands at the WT β_2AR (Figure 4-4B). Most interesting, carbostyril **8** showed the highest affinity of the three ligands at the $S^{5.43(204)}A$ β_2AR mutant; however, its efficacy was not restored by substitution of an alanine for $Ser^{5.43(204)}$. Carbostyril **7** exhibited the greatest loss of affinity at the $S^{5.43(204)}A$ β_2AR mutant, remaining a partial agonist, albeit with a much lower potency than observed at either the WT or the $S^{5.46(207)}A$ mutant β_2AR (Figure 4-4E).

Discussion

The series of experiments described in this chapter confirm that carbostyril **1** interacts with the same agonist-binding pocket as catecholamine ligands (Strader *et al.*, 1997; Strader *et al.*, 1988; Strader *et al.*, 1991; Strader *et al.*, 1989b) and that the 8-hydroxy substituent is cognate to the *para*-hydroxyl group of catechol-based β-agonists. This conclusion rules out the possibility that the significantly higher potency of carbostyril **1** relative to ISO at the β_2AR or the differential dissociation behavior might result from its interaction with an alternate agonist-binding site. Mutation of $Asp^{3.32(113)}$ to glutamic acid significantly decreased the potency of carbostyril **1** at the mutant receptor, consistent with an interaction between this TM domain residue and the protonated amine in the ligand side chain. Simple overlay of carbostyril **1** and ISO suggests that the 8-

hydroxyl substituent and the secondary amide NH are functionally equivalent to the *para*- and *meta*-hydroxyl groups of catecholamine ligands. Preliminary evidence for a hydrogen bonding interaction between the 8-hydroxyl substituent of carbostyril β-agonist and $Ser^{5.46(207)}$ has been provided in recent experiments employing the $S^{5.46(207)}A$ β_2AR mutant and the carbostyril-based β-agonists **9** and **10** (Figure 4-1) (Kikkawa *et al.*, 1997; Kikkawa *et al.*, 1998). That O-methylation of carbostyril **1** (carbostyril **7**) restored affinity at the $S^{5.46(207)}A$ β_2AR mutant, while decreasing potency at the WT β_2AR, provides additional support for the existence of this interaction. Comparison of the structures of carbostyril **7** and **10**, however, reveals that deletion of the hydroxyl group restores ligand binding affinity at the $S^{5.46(207)}A$ β_2AR mutant to an equal degree as addition of a methyl substituent. Given the discrepancy in the steric properties of these two ligands at C-8 of the carbostyril ring, there must be considerable structural flexibility in the β_2AR in this region of the agonist-binding pocket.

Given the apparent functional equivalence of the 8-hydroxyl substituent of carbostyril **1** and the *para*-hydroxyl group of ISO, we sought to establish a hydrogen bonding interaction between the secondary amide NH of the carbostyril ring and the side chain of $Ser^{5.43(204)}$. In agreement with previous studies (Kikkawa *et al.*, 1997), replacement of $Ser^{5.43(204)}$ by alanine caused a larger reduction in affinity of carbostyril **1** for the $S^{5.43(204)}A$ β_2AR mutant than was observed with the $S^{5.46(207)}A$ β_2AR mutant (Table 4-1). These data confirm that the $Ser^{5.43(204)}$ and $Ser^{5.46(207)}$ hydrogen bonds to the carbostyril moiety are not energetically equivalent, in contrast to what is observed for catecholamine-derived β-agonists such as ISO (Strader *et al.*, 1988; Strader *et al.*, 1991).

This inequivalence probably reflects intrinsic differences in polarization of the amide NH and the phenolic OH groups, and hence the carbostyril ring is likely bound within the β_2AR mutant in a similar manner to the catechol portion of ISO.

As the observed reduction of affinity of carbostyril **1** for the $S^{5.43(204)}A$ β_2AR mutant was consistent with the existence of a $Ser^{5.43(204)}$/NH hydrogen bond, we anticipated that carbostyril **8**, obtained by N-methylation of carbostyril **1**, would exhibit enhanced binding affinity for the $S^{5.43(204)}A$ β_2AR mutant in an analogous fashion to that observed for the O-methylated **7** at the $S^{5.46(207)}A$ mutant receptor. The N-methylated carbostyril **8**, although binding more tightly to the $S^{5.43(204)}A$ β_2AR mutant than either carbostyril **1** or **7**, did not, surprisingly, exhibit similar affinity for this mutant receptor to that observed for carbostyril **1** at WT β_2AR. In addition, carbostyril **8** did not induce cAMP formation at the WT β_2AR or either of the $S^{5.43(204)}A$ and $S^{5.46(207)}A$ β_2AR mutants. We have confirmed that carbostyril **8** is able to antagonize cAMP formation when recombinant WT β_2AR is stimulated with ISO (data not shown). These observations imply that simple N-methylation may result in carbostyril **8** binding to the β_2AR in a manner different to carbostyril **1**. Evidence supporting such a proposal is that the decrease in affinity of the N-methylated carbostyril **8** at the $S^{5.46(207)}A$ β_2AR mutant is less than that expected (given that both putative hydrogen bonding interactions have been disrupted) based on the observed affinity changes of either (i) the carbostyril β-agonist **1** interacting with the WT β_2AR or (ii) the 8-methoxycarbostyril derivative **7** at the $S^{5.43(204)}A$ β_2AR mutant (Table 4-1). A less likely explanation of the β_2-antagonist properties of carbostyril **8** might be that carbostyril β-agonist **1** may bind within the WT β_2AR in its tautomeric

form **1a** (Figure 4-1). The presence of the N-methyl group prevents carbostyril **8** being able to exist as a hydroxyquinoline. Further experiments to define the actual binding site of the N-methylated carbostyril derivative **8** in WT β_2AR, and thereby resolve this question may be the subject of future work.

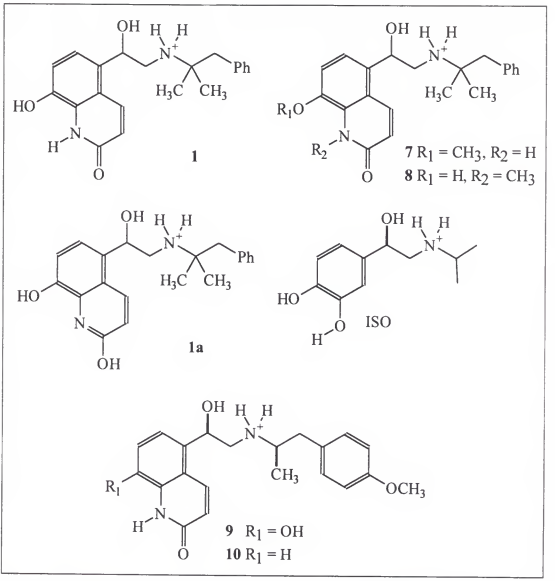


Figure 4-1. **Chemical Structures of carbostyril-based β-agonists 1, 3-6 and ISO.** The compounds were synthesized by Dr. Malgorzata Deyrup using procedures described in Chapter 2 and partially outlined in Figure 4-2.

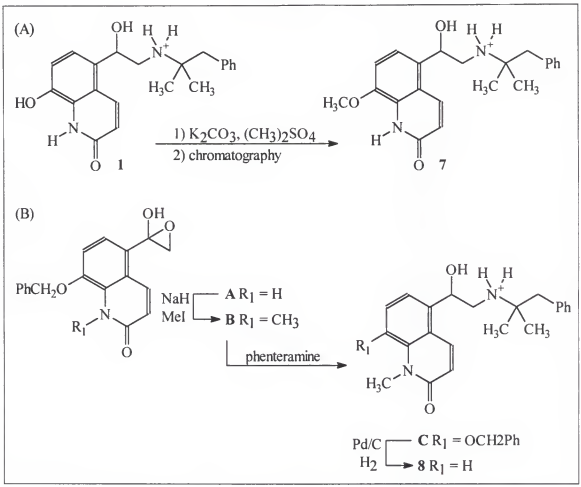


Figure 4-2. **Synthetic Scheme of Carbostyril 7 and 8.** (A) Preparation of O-methylated analog carbostyril **7**. (B) Preparation of N-methylated analog, carbostyril **9** in three steps from known epoxide **A** (Milecki *et al.*, 1987). The syntheses were carried out by Dr. Malgorzata Deyrup using procedures described in Chapter 2.

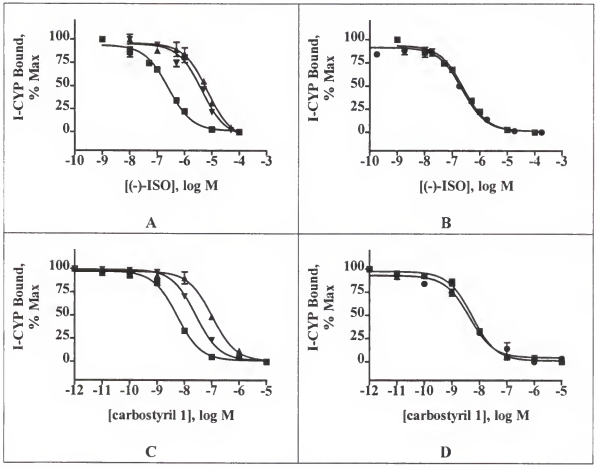


Figure 4-3. **Effect of serine mutations on the binding of ISO and carbostyril 1.** Murine L cells were stably transfected with either the WT β_2AR (■) or the $S^{5.43(204)}$A (▲), $S^{5.46(207)}$A (▼), or $S^{4.53(161)}$A (●) mutant β_2ARs constructs, membranes were prepared and competition binding assays performed in the presence of 100 μM Gpp(NH)p and the indicated concentrations of ISO (Panels A and B) or carbostyril 1 (Panels C and D) , as described under Chapter 2. Data are shown as the mean ± S.E.M. for three or four experiments; see also Table 4-1.

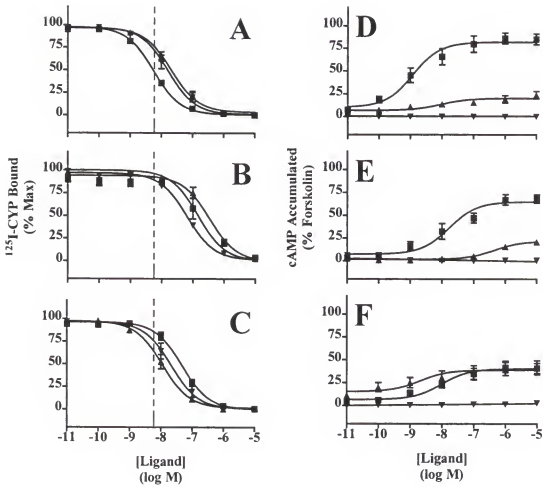


Figure 4-4. **Competition Binding and Functional Characteristics of site-directed mutants of the β_2AR.** Data are shown as the mean ± S.E.M. for three (competition assay) or five (cAMP accumulation) experiments; see also Table 4-1. In panels A-C, membranes from murine L cells stably expressing the WT β_2AR (A), the $S^{5.43(204)}$A β_2AR mutant (B) and the $S^{5.46(207)}$A β_2AR mutant (C) were prepared and competition binding assays performed in the presence of 100 μM Gpp(NH)p and the indicated concentrations of carbostyril **1** (■), **7** (▲) and **8** (▼) as illustrated. In Panels D-F, Whole murine L cells stably expressing the WT β_2AR (D), the $S^{5.43(204)}$A β_2AR mutant (E) and the $S^{5.46(207)}$A β_2AR mutant (F) were assayed for agonist-stimulated adenylyl cyclase activity using carbostyril **1** (■), **7** (▲) and **8** (▼) determined, as described under "Materials and Methods". Results are presented as the fraction of maximal cAMP accumulated in the presence of 1 mM forskolin. The maximal Forskolin-stimulated cAMP accumulation at the WT β_2AR, the $S^{5.43(204)}$A β_2AR mutant and the $S^{5.46(207)}$A β_2AR mutant was 300 ± 30, 250 ± 30, and 420 ± 60 pmol/mg protein/10 min, respectively.

CHAPTER 5
STRUCTURE-ACTIVITY RELATIONSHIPS OF SLOW DISSOCIATING CARBOSTYRIL-BASED AGONISTS FROM THE β_2ADRENERGIC RECEPTOR

Introduction

In the present report, a structure-activity profile for a series of β-agonists was undertaken in order to elucidate the ligand structural requirements that contribute to high affinity for and slow dissociation from the β_2AR. The agonists synthesized by Dr. Malgorzata Deyrup consisted of 8-hydroxycarbostyril derivatives with varying substituents on the side chain amino group. In addition, the contribution of the 8-hydroxycarbostyril nucleus to slow dissociation was investigated by replacement with a catechol ring. Using DDT_1 MF-2 cells that express the β_2AR (Norris *et al.*, 1983), the agonist structures are related to their apparent dissociation rates (indirectly estimated by the rate of cAMP decline), inhibition binding constants (K_i) and by ligand-induced receptor reductions determined by radioligand binding. This information is very valuable to the understanding of the structural requirements of this effect.

Results

Synthesis of β-agonists. The synthetic scheme used by Dr. Malgorzata Deyrup for the 8-hydroxycatbostyril derivatives is shown in Figure 5-1. Synthesis of epoxide **L** was performed using a modified procedure originally described by Milecki *et al.* (1987). Commercially available 8-hydroxyquinoline was oxidized quantitatively to 8-

hydroxyquinoline N-oxide **D**, which was converted in a two-step sequence to 8-hydroxycarbostyril **F** with excellent yield. Substitution at the 5-position of the aromatic ring with glyoxylic acid followed by further functionalization led to 8-benzyloxy-5-oxiranyl carbostyril **L** that served as the starting material for epoxide ring opening reactions with a variety of amines. Compound **M** was prepared using epoxide **L** (Figure 5-1) and phenteramine, which after catalytic hydrogenolysis gave carbostyril **1**. The synthesis scheme for the catecholamine epoxide **T** was performed according to the procedure described by Marki *et al.* (1988). The epoxide was reacted neat with phenteramine and the adduct purified by column chromatography. In the final step, the two benzyl protecting groups were removed by hydrogenolysis using 1,4-cyclohexadiene as a hydrogen source (Figure 5-2).

Carbostyril **1** was originally synthesized by Yoshizaki *et al.* (1976) and reported to be a potent β_2-agonist in isolated tissue preparations. Subsequently, this compound was shown to have a high affinity for the β_2AR in reticulocyte membranes (Standifer *et al.*, 1987). In preliminary experiments, we observed that carbostyril **1** produced antagonist-insensitive stimulation of cAMP accumulation similar to carbo-amine, carbostyril **2** (see Chapter 1). Using carbostyril **1** as the prototype (Figure 5-1), we focused on altering different segments of the side chain phenteramine to assess the effect of these structural modifications on affinity for and slow dissociation from the β_2AR. In derivative **11**, the phenteramine in replaced with propylamine whereas compounds **12** and **6** lack only the geminal methyls or the phenyl ring, respectively. The number of methylene spacers between the carbon bearing the geminal methyl groups and the phenyl ring was changed

to zero (**13**) and three (**14**). In compound **15,** the amino function carries an isopropyl moiety rendering the side chain analogous to ISO. Finally, in derivative **16**, catechol replaced the 8-hydroxycarbostyril nucleus of compound **1** to investigate if this ring contributes to slow dissociation.

β-Agonist effects of synthesized compounds. The effects of the synthesized compounds and ISO on stimulated cAMP accumulation in DDT cells is shown in Table 5-1. The EC_{50} values for cAMP accumulation varied from a low of 0.5 nM for **1** to a high of 34.8 nM for **13**. The overall potency series is **1** > **14** > **12** > **15** = ISO = **16** > **6** > **11** = **13**. The maximal stimulation of cAMP accumulation for all of the synthesized compounds was not different from ISO indicating that they are all full β_2AR agonists (Table 5-1).

Table 5-1.
Agonist properties of β-agonists.

Compound	EC_{50} (nM)	Maximal Response (% of ISO Maximum)
ISO	6.2 ± 0.9 (8)	100
1	0.5 ± 0.1 (9)	104 ± 5
6	15 ± 5 (4)	105 ± 4
11	31 ± 7 (4)	91 ± 4
12	3 ± 1 (7)	105 ± 3
13	35 ± 8 (6)	100 ± 10
14	1.9 ± 0.7 (5)	92 ± 8
15	5 ± 2 (8)	97 ± 4
16	6 ± 2 (7)	92 ± 6

DDT cells were incubated with 50 μM rolipram and varying concentrations of the β-agonists for 10 min at 37 °C. At the end of the incubation, the cAMP accumulated was determined as described in Chapter 2. Each value is the mean ± S.E.M. with the n in parenthesis. The basal and ISO maximal stimulation of cAMP accumulation was 63 ± 14 and 1148 ± 74 pmol/mg per 10 min (n=8).

The stimulation of cAMP accumulation produced by 1 μM of all of the agonists was completely blocked by the concurrent addition of 20 μM propranolol. In addition, none of the agonists (1 μM) inhibited phosphodiesterase activity as determined using whole cell homogenates (data not shown).

Apparent dissociation rate (k_{off}) and K_i for the β-Agonists. A functional assay was designed to estimate indirectly the apparent k_{off} for each of the β-Agonists. In this method, the rate of cAMP decline in DDT cells after stimulation by each agonist alone was determined in the presence of a 20-fold excess of the β-antagonist propranolol. Because cAMP formation depends upon receptor occupancy by the agonists during the initial incubation, the subsequent rate of cAMP decline, likely due to degradation by phosphodiesterases, should be related to the k_{off} of the agonist from the receptor. The accuracy of the relationship between the agonist k_{off} from the receptor and the rate of cAMP decline may partly depend upon the presence of a receptor reserve. If a significant receptor reserve exists, the apparent k_{off} will be overestimated due to a nonlinear relationship between receptor occupancy and response. In order to ensure that cAMP accumulation was related to receptor occupancy, initial experiments were performed to determine if a receptor reserve for either ISO or carbostyril **1** stimulated cAMP accumulation was present in DDT cells. Cells were pretreated with varying concentrations of the irreversible β-antagonist DCITC, washed 15 times to remove unbound ligand, and the maximal cAMP accumulation by ISO and carbostyril **1** was determined. The concentration range of DCITC used (0.25-10 nM) reduced the receptor content from 25 to 78 % (as determined by Rosenthal plots of [^{125}I]-CYP binding)

without a change in the K_D value for this radioligand (data not shown). As illustrated in Figure 5-3, a hyperbolic relationship was observed between receptor occupancy and maximal response. The receptor reserve for ISO and carbostyril **1** was calculated to be 32 and 25 %, respectively because the maximal stimulation of cAMP accumulation (defined as > 90 %) was achieved at receptor occupancies of 68 % for ISO and 75 % for carbostyril **1**. Therefore, in experiments to determine the apparent koff for the agonists, the receptor content was decreased 40- 50 % (to delete the receptor reserve) by initially pre-treating the cells with 1 nM DCITC followed by washing.

Figure 5-4A shows the effect of ISO on cAMP accumulation of DCITC pretreated DDT cells in the absence of a phosphodiesterase inhibitor. This agonist (1 μM) produced a rapid increase in cAMP accumulation reaching a maximum after 1 min of incubation. The maximum stimulation cAMP accumulation was maintained within 10 % during the next 9 min of incubation. Over the 10 min incubation period, less than 10 % of the cAMP accumulated was extracellular and with longer incubation periods the levels of cAMP gradually declined (data not shown). The addition of propranolol (20 μM) after 4 min of incubation with ISO resulted in a rapid decline of cAMP which approached basal levels within 1.5 min. Figure 5-4B depicts the rate of decline of cAMP after the addition of propranolol for several of the agonists while Table 5-2 lists the apparent k_{off} calculated from the rate of cAMP decline. The apparent k_{off} order is ISO = **11** > **13** > **6** > **16** > **15** > **12** > **14**. An apparent k_{off} value for carbostyril **1** could not be calculated because no significant cAMP decline was observed over the propranolol incubation period (Figure 5-4B).

Table 5-2.
Agonist apparent k_{off} and K_I values.

Compound	k_{off} (apparent) (min^{-1})	K_I (nM)
ISO	5.5 ± 0.3 (8)	117 ± 2 (3)
1	Not Detectable	1.6 ± 0.2 (6)
6	3.4 ± 0.6 (4)	37 ± 1 (4)
11	5.4 ± 0.4 (3)	300 ± 30 (5)
12	0.21 ± 0.05 (5)	21 ± 2 (4)
13	4.6 ± 0.8 (4)	80 ± 20 (4)
14	0.05 ± 0.01 (4)	4.5 ± 0.7 (3)
15	2.0 ± 0.5 (6)	27 ± 3 (4)
16	4.1 ± 0.6 (5)	80 ± 20 (4)

The apparent K_{off} was calculated as the rate of cAMP decline in 1 nM DCITC pretreated DDT cells. After a 4 min stimulation of cAMP accumulation, by 1 μM of each agonist, propranolol (20 μM) was added and the cAMP determined as a function of time. The K_I values were determined from displacement of [^{125}I]-CYP binding in the presence of 100 μM Gpp(NH)p. Each value is the mean ± S.E.M. with the n in parenthesis.

Although the receptor reserve was eliminated for the determination of the dissociation rates reported in Table 5-2, parallel experiments performed using untreated cells resulted in similar values for the dissociation rate of carbostyril **6**. Therefore, the data suggest that the receptor reserve is not large enough to effect the accuracy of the k_{off} values (data not shown).

Table 5-2 also shows the calculated K_I values for the agonists determined from displacement of [^{125}I]-CYP binding to the β_2AR in DDT cell membranes in the presence of 100 μM Gpp(NH)p. These K_I values ranged from a low of 1.6 nM for **1** to a high of 300 nM for **11**. The K_I for **1** did not change by increasing the incubation time beyond the 60 min used. Figure 5-5 depicts the relationship between the apparent k_{off} and K_I values for the agonists. Linear regression analysis using the data from all of the agonists (except **1**) gave and r^2 of 0.51. However, the correlation between the apparent k_{off} and K_I values

for the agonists was increased ($r^2 = 0.87$) when compound **11** was not included in the analysis.

Effect of agonists on the β_2AR content of DDT cell membranes. To further determine if the agonists tightly bind to the β_2AR, their effect to produce an apparent receptor loss was assessed. As shown in Figure 5-6A, pretreatment of DDT cell membranes with 1 μM ISO or **6** in the presence of 100 μM Gpp(NH)p for 4 min at 37 °C followed by 6 membrane wash cycles, did not alter either the B_{max} or K_D value for [^{125}I]-CYP binding to the β_2AR. In contrast, pretreatment with carbostyril **1** (1 μM for 4 min at 37 °C), reduced maximal [^{125}I]-CYP binding by 68 %. The K_D for [^{125}I]-CYP binding to the β_2AR was not changed after the pretreatment with the agonists followed by membrane washing (control, 31 pM; carbostyril **1**-pretreated, 34 pM). Figure 5-6B shows the effect of the agonists on [^{125}I]-CYP binding after a 4 min incubation with DDT cell membranes in the presence of 100 μM Gpp(NH)p followed by 6 membrane wash cycles. No change in [^{125}I]-CYP binding was observed after pretreatment with ISO, **11**, **6**, **13**, **15** and **16**. A 59 and 4 % reduction in [^{125}I]-CYP binding occurred after pretreatment with **1** and **14**, respectively, whereas a 13 % reduction occurred after **12** pretreatment.

Discussion

The goal of the present study was to determine the structural requirements for 8-hydroxycarbostyril-based β-agonists which contribute to high affinity for and slow-dissociation from the β_2AR. Using carbostyril **1** as the prototype high affinity and slow dissociating agonist, the 8-hydroxycarbostyril nucleus and several side chain substituents were identified as important contributors to the unique binding properties of this

compound. All of the derivatives synthesized stimulated cAMP accumulation in DDT cells with potencies ranging from the sub- to mid-nanomolar range and with a maximal stimulation that was not different from ISO. Furthermore, the stimulation of cAMP accumulation by each of the derivatives and ISO was blocked by the concurrent addition of the β-antagonist propranolol. Taken together, these data indicate that the response by each of the compounds is mediated by the βAR and that each acts as full agonists.

In this study, the agonist structures were related to their estimated dissociation rates (k_{off}), K_i values for the β_2AR and their ability to induce a decrease in the receptor content of the DDT cell membranes. In order to estimate the agonist k_{off} from the β_2AR, a functional assay was developed where the rate of cAMP decline was determined in the presence of excess β-antagonist. Since cAMP accumulation is dependent upon receptor occupancy, the rate of cAMP decline after addition of antagonist should approximate the agonist k_{off} from the receptor. Cellular functional assays have been precisely used to estimate the rate constants for receptor ligands (Sklar *et al.*, 1985; Mueller *et al.*, 1988). It should be pointed out however, that the estimated k_{off} might be affected by several factors including the presence of a receptor reserve as well as agonist-induced desensitization of the response. In addition, the intrinsic rate of cellular cAMP decline (rate of cAMP degradation) would provide the upper limit in estimating high k_{off} values. A high receptor reserve or non-proportional relationship between receptor occupancy and response could result in an underestimate of the koff. Using the irreversible antagonist DCITC, a 25 and 32 % receptor reserve for ISO and carbostyril **1**, respectively, was found for the maximal stimulation of cAMP accumulation in DDT cells. Therefore, to improve the accuracy of

the k_{off} determination, the cells were pretreated with the irreversible β-antagonist DCITC to reduce the receptor content (receptor reserve) and provide a more proportional relationship between receptor occupancy and response. However, there was no difference in the rate of decline of carbostyril **6** between using treated and untreated groups of DDT cells. Therefore, the theoretical effect of a receptor reserve on the k_{off} determination using this method may be greatly exaggerated. Desensitization of the β_2AR in DDT cells has been reported (Scarpace *et al.*, 1985; Strasser *et al.*, 1986) and may have occurred during the time course that cells were exposed to agonists (10 min). However, the observation that the maximal accumulation of cAMP declined less than 10 % indicated that substantial stimulation of cAMP accumulation was sustained during the agonist exposure time. With incubation periods longer that 10 min, desensitization likely became more significant because the maximal cAMP accumulated declined. Therefore, estimation of any dissociation of carbostyril **1** from the β_2AR would prove to be inaccurate.

The addition of propranolol following the initial stimulation of cAMP accumulation with ISO resulted in a rapid decline in cAMP. The calculated k_{off} based upon the rate of decline was 5.5 ± 0.3 min^{-1}. This off-rate corresponds to a half-life of 7.5 sec and is in good agreement with the reported half-life (3-4 sec) for ISO at the β_2AR based upon two independent determinations reported (Mueller *et al.*, 1988; Voss *et al.*, 1992). At the other extreme, the carbostyril **1** mediated increase in cAMP accumulation did not decline after the addition of propranolol indicating that carbostyril **1** was tightly bound to and slowly dissociated from the β_2AR. This observation is consistent with that reported for carbo-amine (carbostyril **2**), a β-agonist structurally similar to carbostyril **1**,

which was shown to produce sustained activation of adenylyl cyclase in reticulocyte membranes (Standifer *et al.*, 1989). The antagonist-insensitive effect of carbostyril **1** was not likely due to non-receptor mediated changes in cAMP accumulation because the increase was blocked by the concurrent addition off propranolol and carbostyril **1** did not inhibit phosphodiesterase activity at the concentration used. For several reasons, the tight binding of carbostyril **1** to the β_2AR is probably not dependent on ternary complex formation involving the agonist, receptor and G_s (De Lean *et al.*, 1980). First, the cAMP determinations were performed in intact cells where ternary complex formation is likely to be transitory due to the presence of endogenous guanine nucleotides. Second, pretreatment of DDT cell membranes with carbostyril **1** in the presence of Gpp(NH)p, to maintain the receptor in an agonist low affinity state, resulted in a apparent loss of β_2AR content. Finally, competition curves of carbostyril **1** with [^{125}I]-CYP in the presence and absence of Gpp(NH)p resulted in no shift in affinity indicating that high affinity agonist binding is present independent of an interaction with G-protein.

A good correlation between the K_I and functionally derived k_{off} was obtained for the agonists when carbostyril **11** was excluded from the analysis. This correlation suggested that the k_{off} values obtained from the functional assay are a good approximation of the agonists k_{off} from the receptor. The correlation indicated that k_{on} for the various agonists is similar and that k_{off} is the major factor determining the differences in their K_I values because the K_I of the agonists is a function of k_{off}/k_{on}. The estimated k_{on} was calculated to be 5 x 10^7 $min^{-1}M^{-1}$. Carbostyril **11** had an estimated k_{off} similar to ISO but a higher K_I value. Assuming similar association rates for ISO and carbostyril **11** for the

β_2AR, then carbostyril **11** would be predicted to have a higher k_{off} as compared to ISO. If extrapolation of the linear regression is valid, the dissociation rate of carbostyril **11** would be 15 min^{-1} ($r^2 = 0.86$). Therefore, the lower apparent k_{off} for carbostyril **11** than expected may be due to this agonist having a faster k_{off} from the receptor than the intrinsic rate of cAMP decline. Compound **1** was not included in the analysis because its antagonist-insensitive stimulation of cAMP accumulation precluded the calculation of an apparent k_{off}. Nonetheless, carbostyril **1** had the lowest K_1 of the agonists, which is consistent with its apparent tight binding to the receptor as indicated in the functional assay.

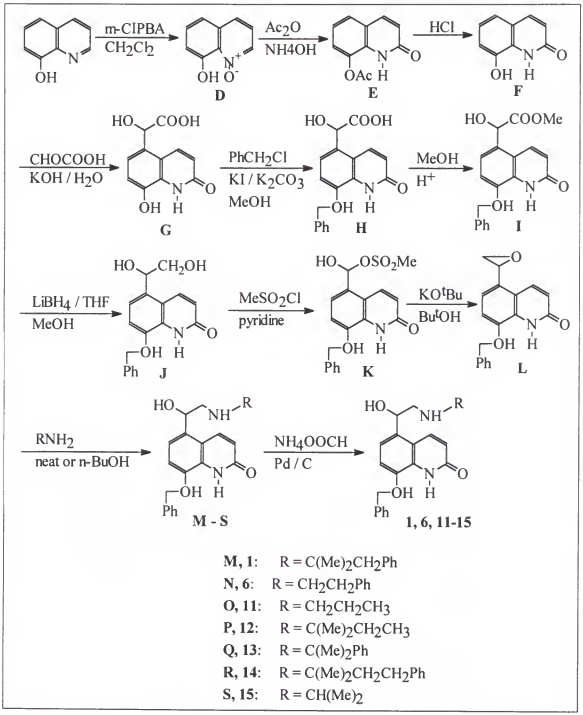
Based upon the estimated k_{off} and K_1 values for the agonists, several structural characteristics of carbostyril **1** emerged that are required for its high affinity and slow dissociation from the β_2AR. The relatively high apparent k_{off} and K_1 values for the side chain amino derivatives comprising propyl (**11**), 2-methylbutyl (**12**), phenethyl (**6**) and isopropyl (**15**) indicated that the side chain geminal methyls and phenyl groups are necessary for the slow dissociation observed with **1**. The distance between the geminal methyls and phenyl group also appears to contribute to high affinity and slow dissociation. In carbostyril **1**, a single methylene separated the geminal methyls and the phenyl group. When the number of methylenes was reduced to zero, as in compound **13**, the estimated k_{off} and the K_1 were relatively high. When the number of methylenes was increased to three (carbostyril **14**), the estimated k_{off} was relatively low but dissociation was still faster than with carbostyril **1**. Finally, replacing the 8-hydroxycarbostyril nucleus of carbostyril **1** with the catechol moiety (compound **16**) resulted in an agonist with a relatively higher k_{off} and K_1 value. This suggested that the 8-hydroxycarbostyril

nucleus in conjunction with the side chain geminal methyls and phenyl group are contributing factors to the slow dissociation and low K_I of carbostyril **1**. It has been suggested (Yoshizaki *et al.*, 1976; Kikkawa *et al.*, 1997) and verified (see Chapter 4) that the hydroxyl and amino groups of 8-hydroxycarbostyril correspond to the *para*- and *meta*-hydroxyl groups of catecholamines, respectively, and interact with the same serine residues in the β_2AR. Interestingly, replacement of the 8-hydroxycarbostyril of carbostyril **1** with catechol (compound **16**) reduces the affinity of the latter by 48-fold. Although the side chain substituents for both compounds remain identical. This would indicate that the 8-hydroxycarbostryil nucleus contributes significantly to the overall affinity of carbostyril **1** through distinct interactions with the receptor unavailable to the catechol moiety. These may include interactions with the carbonyl or enhanced interactions between the receptor and the ring system. Additionally, the hydrogen bond formed between the hydroxyl group of the receptor and the amide proton on the 8-hydroxycarbostyril may be stronger in character due to the acidic nature of the amide proton compared to a catechol hydroxyl.

The structure-activity profile based upon the estimated k_{off} values was largely confirmed using radioligand binding. Thus, pretreatment of DDT cell membranes with carbostyril **1**, **14** and **12** that had the lowest apparent k_{off} values in the functional assay produced a decrease in [^{125}I]-CYP binding to the β_2AR. This decrease was likely due to tight binding of these agonists to the receptor. None of the other agonists, which had relatively high apparent k_{off} values, produced a decrease in radioligand binding indicating that they rapidly dissociated from the receptor.

A complete loss of [^{125}I]-CYP binding was not observed with either carbostyril **1**, **14** or **12** pre-incubations. This may be due to incomplete slow dissociation of the ligands from the β_2AR during the 30 min incubation at 37 ^{O}C with [^{125}I]-CYP. However, a significant portion of the receptors (60 %) remains bound with carbostyril **1** after this period. Therefore, the half-life of carbostyril **1** appears to be greater than 30 min (k_{off} < 0.02 min –1). Although our data indicates that the side chain geminal dimethyl and phenyl groups are involved in the ligand structural requirements for slow dissociation, other side chain modifications may also suffice. For example, TA 2005 (carbostyril **9**) another 8-hydroxycarbostyril-based β-agonist which contains a single methyl on the alpha carbon to the amino group and a *para*-methoxyphenyl on the side chain was shown to produce a sustained β-agonist response in an isolated tissue suggesting that it is a slow dissociating β-agonist (Voss *et al.*, 1992). The high-affinity binding of TA-2005 was also shown to be mediated by a specific interaction with the seventh TM domain of the β_2AR (Kikkawa *et al.*, 1998). In comparison to carbostyril **1**, the selective β_2-agonist salmeterol has been shown to produce sustained activation of the β_2AR that has been shown to involve anchoring of the molecule's side chain to a receptor exosite (Green *et al.*, 1996). The receptor exosite has been shown using site-directed mutagenesis to structurally correspond to a discrete region of the fourth TM domain of the β_2AR. A gain in exosite binding in the β_1AR was conferred without changing the affinity of salmeterol for the β_1AR. In the case of salmeterol, there appears to be dissociation between the increased affinity observed for the β_2AR and the long duration of action of salmeterol at the β_2AR.

Carbostyril **1** appears to be an additional research tool to probe the structural features of the β_2AR that may lead to slow-dissociation of agonists. It remains unclear whether the slow-dissociation of carbostyril **1** is purely a thermodynamic effect and undissociable form its high-affinity binding or whether specific interactions exists between the side chain of carbostyril **1** and a receptor exosite.



M, 1: R = $C(Me)_2CH_2Ph$
N, 6: R = CH_2CH_2Ph
O, 11: R = $CH_2CH_2CH_3$
P, 12: R = $C(Me)_2CH_2CH_3$
Q, 13: R = $C(Me)_2Ph$
R, 14: R = $C(Me)_2CH_2CH_2Ph$
S, 15: R = $CH(Me)_2$

Figure 5-1. **Synthetic scheme for 8-hydroxycarbostyril derivatives.**

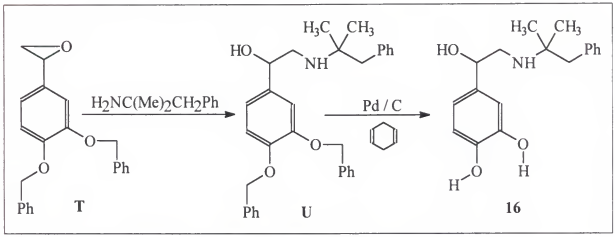


Figure 5-2. **Synthetic scheme for catecholamine derivative.**

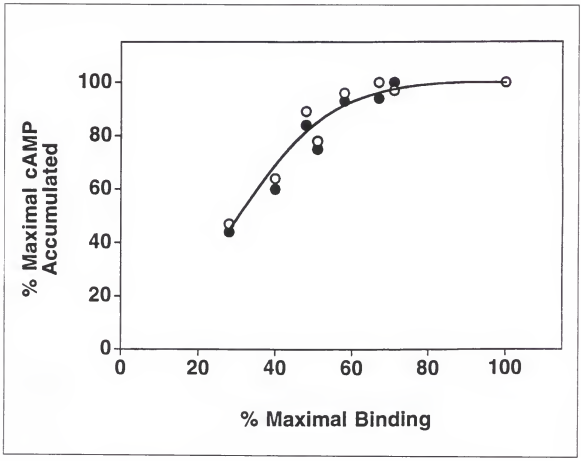


Figure 5-3. **Relationship between βAR occupancy and maximal response in DDT cells.** Attached DDT cells were incubated without and with 0.25, 0.5, 0.75, 1.0, 2.5, 5 and 10 nM DCITC for 20 min at 37 ^{O}C and then washed 15 times in HBSS. Detatched cells were then incubated with 50 μM rolipram and with or without 10 μM ISO (O) or 10 μM carbostyril **1** (●) for 10 min at 37 ^{O}C. At the end of the incubation, the cAMP accumulated was determined as described in Chapter 2. Parallel samples of DCITC-pretreated cells were homogenized, membranes isolated and the βAR content was determined by the specific binding of 100 pM [^{125}I]-CYP. The control ISO and carbostyril **1** stimulated cAMP accumulation was 1262 ± 174 and 1057 ± 150 pmol / mg protein / 10 min, respecitvely (mean ± S.E.M., n=4-6).

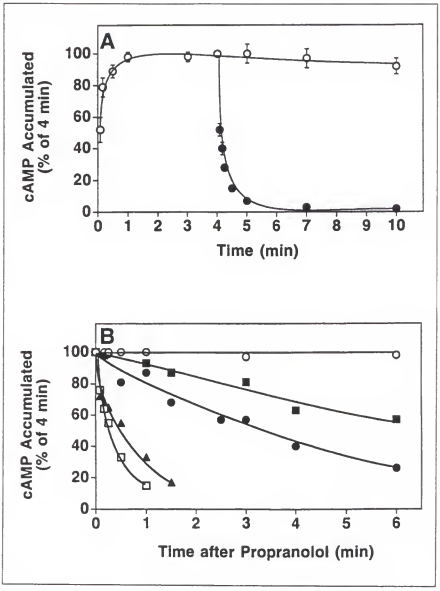


Figure 5-4. **Time course of cAMP accumulation and decline in DDT cells.** In panel A, suspensions of DDT cells were incubated with 1 μM ISO at 37 ^{O}C and at the times indicated, the cAMP accumulated was determined (O). After 4 min of incubation, propranolol (20 μM) was added to parallel samples and the cAMP accumulated was measured as a function of time (●). The basal and ISO-stimulated cAMP accumulated at 4 min was 31 ± 8 and 680 ± 40 pmol / mg protein (mean ± S.E.M., n = 7). In panel B, cells were incubated with 1 mM of carbostyril **1** (O), **6** (▲), **12** (●), **14** (■) or **15** (□) for 4 min at 37 ^{O}C. Propranolol (20 μM) was then added (time = 0) and the cAMP decline was determined at the times indicated.

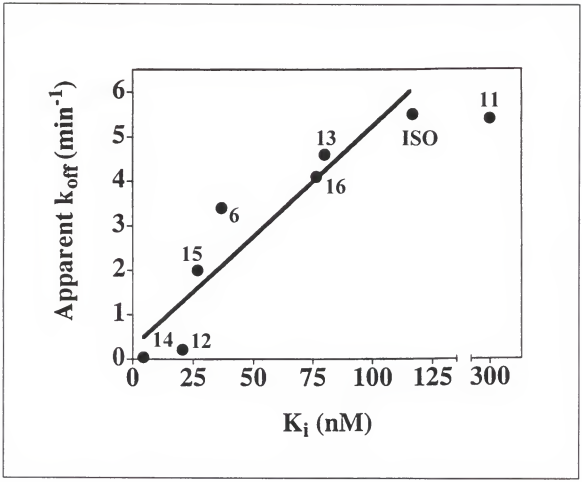


Figure 5-5. **Linear regression analysis of the relationship between the apparent k_{off} and K_I.** The regression line includes data taken from Table 5-2 with the exception of carbostyril **11**.

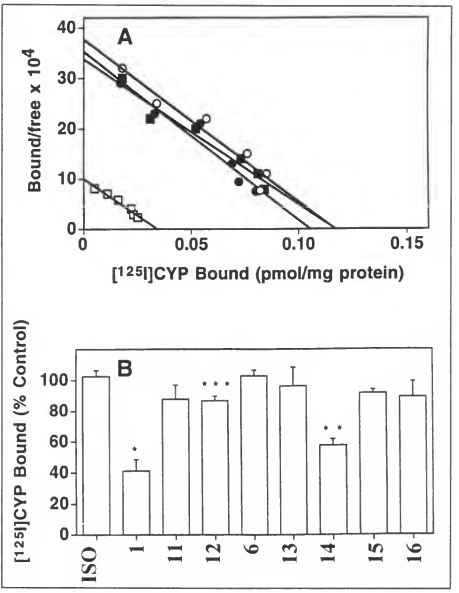


Figure 5-6. **Effect of β-agonist pretreatment on βAR content of DDT cell membranes.** In panel A, membranes were incubated with 100 μM Gpp(NH)p and without (O) or with 1 μM ISO (●), carbostyril **1** (□) or carbostyril **6** (■) for 4 min at 37 ^{O}C. The membranes were then washed 6 times and the specific binding of 6-100 pM [^{125}I]-CYP (45 min at 36 ^{O}C) was determined. Data is representative of 3 separate determinations. In panel B, membranes were incubated in the presence of 100 μM Gpp(NH)p and with or without 1 μM of the indicated agonist for 4 min at 37 ^{O}C. The membranes were then washed 6 times and specific binding of 100 pM [^{125}I]-CYP (30 min at 36 ^{O}C) was determined. Data is the mean ± S.E.M. of 3-5 separate experiments. The control [^{125}I]-CYP binding ranged from 85-179 fmol/mg protein. * $p < 0.005$. ** $p < 0.01$, *** $p < 0.05$ compared to control.

CHAPTER 6
CHARACTERIZATION OF THE STRUCTURAL REQUIREMENTS OF THE SUBTYPE SELECTIVE SLOW DISSOCIATION KINETICS USING CHIMERIC β_1/β_2 ADRENERGIC RECEPTORS

Introduction

The structural characterization of the complexes between G protein-coupled receptors (GCRs) and their ligands has been the subject of much research over the past decade (Ballesteros and Weinstein, 1995; Strader *et al.*, 1994; Oprian, 1992). In combination with experimental methods such as fluorescence spectroscopy (Gether *et al.*, 1997a; Gether *et al.*, 1997b), a number of modeling and dynamic simulations have yielded substantial insight into the molecular basis of ligand selectivity (Kontoyiani and Lybrand, 1993; Trumpp-Kallmeyer *et al.*, 1993; Gouldson *et al.*, 1997), and agonist or antagonist activity (Zhang and Weinstein, 1993; Luo *et al.*, 1994). An essential element in performing such theoretical studies is knowledge of the residues defining the binding site of the agonist or antagonist of interest (Ballesteros and Weinstein, 1995). The β_2AR is an almost ideal system for investigating the molecular details of ligand recognition and receptor activation, given recombinant DNA methodology for the expression of the WT β_2AR, site-directed β_2AR mutants and chimeric receptors (Kobilka *et al.*, 1987; Kobilka *et al.*, 1988; Frielle *et al.*, 1988; Mizobe *et al.*, 1996). In addition, because of the medical importance of compounds that act at βARs, a variety of classes of agonists and antagonists have been described (Hieble *et al.*, 1995; Ruffolo *et al.*, 1995). This level of

experimental characterization has provided a firm foundation for the construction, and evaluation, of molecular models of the TM domains of the β_2AR that seek to provide a rational basis for ligand selectivity (Gouldson *et al.*, 1997; Donnelly *et al.*, 1994; Maloney-Huss and Lybrand, 1992; Kontoyianni *et al.*, 1996).

A novel class of long acting antagonist-insensitive β_2AR agonists is emerging which further addresses the issue of receptor-ligand selectivity. The compound investigated in this study is a member of the carbostyril-based β–agonists that have been shown to be a highly potent and efficacious agonists at the βARs (manuscript submitted, Chapter 5). Prior to these studies, it has been shown that salmeterol, a catecholamine-based β–agonist, exhibits a long duration of action at the β_2AR. This agonist was of significant interest given that it addressed an inherent limitation of many GCR-agonists, which was the rapid dissociation from target receptors (Johnson, 1995; Ball *et al.*, 1991). Therefore, a single exposure to salmeterol causes persistent activation of β_2ARs, both in tissue and cell culture. However, addition of an antagonist inhibits receptor activation, presumably by displacing salmeterol from the agonist-binding pocket. Most significantly, the agonist activity is reasserted after removal of the antagonist from the β_2AR (Nials *et al.*, 1993). These results implied that salmeterol interacted with an additional exosite on the β_2AR that prevents ligand dissociation from the receptor, although the moiety associated with agonist activity can be displaced from its binding pocket by antagonist. Support for such a hypothesis, and the identification of the residues comprising this exosite, has been demonstrated in a series of elegant experiments employing β_1/β_2ARs TM IV chimera/mutants (Green *et al.*, 1996). However, it has not been shown how these

residue differences result in alteration of the subtype selective long term binding of salmeterol or whether other exosites may exist within the β_2AR that can confer such an activity. It was our aim to identify the region responsible for the subtype selective, antagonist insensitive slow dissociation of this carbostyril-based β-agonist from the β_2AR to determine if a common exosite is required for slow dissociation from the β_2AR.

With a firm understanding of the structural basis of subtype-selective, slow dissociation at the β_2AR, the design of additional compounds with extended duration of action can be contemplated. Before such development can occur, the specific interactions leading to the enhanced duration of action of other ligands must be identified. To gain greater insight into the structural nature of slow dissociation and to show whether this particular exosite is generally responsible for slow dissociation additional information must be obtained. It is with this perspective that we now report new data that describes a novel carbostyril-based β-agonist that exhibits a subtype-selective, enhanced duration of action that arises from differences in ligand dissociation behavior at the β_1AR and β_2AR. More importantly, the enhanced duration of action appears to be antagonist insensitive in contrast to that observed for salmeterol. Following the characterization of the dissociation behavior of the carbostyril-based β-agonist **1** at a series of chimeric and mutant β_1/β_2ARs, a discrete region of the TM IV domain of the β_2AR has been implicated as the principle structural determinant underlying its subtype-selective, antagonist-insensitive enhanced duration of action. This region is distinct from that which represents the salmeterol exosite.

Results

In competition-binding experiments, the racemic carbostyril **1** was a potent β_2-agonist with an apparent K_i of 3.2 ± 0.2 nM at recombinant WT β_2AR expressed in murine L cells (Table 6-2, Figure 6-2A). For comparison, the apparent K_i of ISO was 260 ± 40 nM under our assay conditions. We measured the dissociation behavior of carbostyril **1** and ISO at both the WT β_1 and β_2ARs using an indirect assay. In this approach, whole cells were stimulated with 1 μM of either carbostyril **1** or ISO for 4 minutes to achieve steady state cAMP accumulation. The addition of 20 μM propranolol at this time was used to assess the sensitivity of the binding of either carbostyril **1** or ISO to the presence of antagonist. The time-dependent decay in cAMP levels, after antagonist addition was then monitored and used to derive the apparent dissociation rate of the β-agonist (Figure 6-4, Table 6-1).

Table 6-1.
Dissociation rates of carbostyril 1 and ISO from βAR constructs.

Construct	k_{off} (min^{-1}) Carbostyril **1**	k_{off} (min^{-1}) ISO
WT β_1AR	1.7 ± 0.4	9 ± 3
β_2(I)/β_1(II-VII)AR	3 ± 2	17 ± 5
β_2(I-II)/β_1(III-VII)AR	1.3 ± 0.4	8 ± 2
β_2(I-III)/β_1(IV-VII)AR	2.0 ± 0.8	11 ± 4
β_2(I-IV)/β_1(V-VII)AR	0[a]	50 ± 20
β_2(I-V)/β_1(VI-VII)AR	0[a]	30 ± 20
β_2(I-VI)/β_1(VII)AR	0[a]	4 ± 2
WT β_2AR	0[a]	17 ± 7
β_2S(149-173)AR	1.1 ± 0.2	24 ± 6
β_2S(149-166)AR	0.5 ± 0.1	30 ± 10
β_2S(164-173)AR	0[a]	20 ± 8
β_2S(149-158)AR	0[a]	24 ± 6
β_1S(177-181)AR	2.6 ± 0.5	21 ± 8

[a] No detectable dissociation occurred during the time course of the experiment.

To validate our measurements, the dissociation rates of ISO from the WT β_1 and β_2ARs (9 ± 3 and 17 ± 7 min^{-1}, respectively) were found to be in good agreement with previous measurements (10 - 13 min^{-1}) at the WT β_2AR (Mueller *et al.*, 1988; Voss *et al.*, 1992). The addition of propranolol to cells expressing the WT β_2AR pretreated with carbostyril **1** resulted in no detectable decrease in the steady state accumulation in cAMP. In contrast, cells expressing the WT β_1AR pretreated with carbostyril **1** showed dramatic decreases in the steady state accumulation of cAMP after the addition of propranolol. The apparent dissociation rate constant for the decay of carbostyril **1** from the WT β_1AR was 1.7 ± 0.4 min^{-1} (Figure 6-4, Table 6-1).

To determine the important TM domain responsible for the subtype selective antagonist insensitivity, we characterized a series of β_1/β_2AR chimeric receptor constructs obtained from Dr. Thomas Frielle, Pennsylvania State University Medical Center, Hershey, PA (Figure 6-1) (Frielle *et al.*, 1988). Each open-reading frame was subcloned into the mammalian expression vector, pcDNA3, and transfected into murine L cells. G-418 resistant colonies were isolated and assayed for specific [^{125}I]-CYP binding. Cells expressing chimeric receptors were characterized using [^{125}I]-CYP saturation binding. The [^{125}I]-CYP binding affinity for the chimeric β_1/β_2AR is listed in Table 6-2. These values are similar to the affinities determined for the WT β_1 and β_2ARs (40 ± 10 and 21 ± 2 pM, respectively). Radioligand competition binding with carbostyril **1** and ISO was performed. The K_i values determined for carbostyril **1** and ISO at each of the chimeric receptors are similar to those determined for the WT β_1 and β_2ARs (Table 6-2 and Figure 6-2).

Table 6-2.
Radioligand binding data.

Construct	[^{125}I]-CYP K_D (pM)	Carbostyril **1** K_I (nM)	ISO K_I (nM)
WT β_1AR	40 ± 10	110 ± 10	170 ± 40
β_2(I)/β_1(II-VII)AR	54 ± 4	142 ± 6	180 ± 30
β_2(I-II)/β_1(III-VII)AR	22 ± 3	50 ± 10	120 ± 20
β_2(I-III)/β_1(IV-VII)AR	27 ± 6	52 ± 4	240 ± 50
β_2(I-IV)/β_1(V-VII)AR	60 ±10	16 ± 2	600 ± 100
β_2(I-V)/β_1(VI-VII)AR	24 ±2	8 ± 1	120 ± 30
β_2(I-VI)/β_1(VII)AR	44 ± 2	40 ± 10	800 ± 100
WT β_2AR	21 ± 2	3.2 ± 0.2	260 ± 40
β_2S(149-173)AR	32 ± 5	1.7 ± 0.1	42 ± 5
β_2S(149-166)AR	21 ± 2	2.5 ± 0.1	160 ± 10
β_2S(164-173)AR	18 ± 4	2.7 ± 0.2	180 ± 40
β_2S(149-158)AR	35 ± 6	3.1 ± 0.5	280 ± 40
β_1S(177-181)AR	46 ± 4	80 ± 40	290 ± 50

The affinity of carbostyril **1** is dramatically enhanced by the addition of TM V of the β_2AR to the β_1AR sequence (β_2(I-V)/β_1(VI-VII)AR), with a modest increase in affinity following the addition of β_2AR TM IV sequence to the chimeras (β_2(I-IV)/β_1(V-VII)AR). This trend did not continue because the affinity of carbostyril **1** at the β_2(I-VI)/β_1(VII)AR chimera was greatly reduced compared to its affinity at the WT β_2AR.

The dissociation behavior of carbostyril **1** and ISO was then determined for each of the chimeric receptors to determine which TM domain is responsible for the subtype selective antagonist-insensitive binding of carbostyril **1** to the β_2AR (Table 6-1 and Figure 6-5). As from the WT β_2AR, the dissociation rate of carbostyril **1** from the β_2(I-IV)/β_1(V-VII), β_2(I-V)/β_1(VI-VII) and β_2(I-VI)/β_1(VII) chimeras was sufficiently slow as to be undetectable over the time period of this assay. In contrast, carbostyril **1** dissociated from the β_2(I)/β_1(II-VII), β_2(I-II)/β_1(III-VII) and β_2(I-III)/β_1(IV-VII) chimeric receptors at apparent dissociation rates identical to that observed the WT β_1AR (Table 6-1). Given

that the observed changes in ligand dissociation behavior might have resulted from gross structural changes in the β_1/β_2AR chimeras, we also demonstrated that no similar variation in dissociation behavior of ISO was observed at each of these β_1/β_2AR chimeric receptors (Table 6-1 and Figure 6-5).

To provide additional evidence for the role of the β_2AR TM IV domain in conferring the subtype selective antagonist-insensitive binding of carbostyril **1** at the β_2AR, several additional β_1/β_2AR chimeric/mutant receptors were characterized (Figure 6-1). These constructs selectively exchange either the entire TM IV domain of the β_2AR or discrete regions of the TM IV domain of the β_2AR with the cognate sequence of the β_1AR (Green *et al.*, 1996). An additional chimera was also characterized. This chimera represents the exchange of a discrete region of the TM IV domain of the β_1AR with the cognate sequence of the β_2AR. The [^{125}I]-CYP affinities for each of these chimeras were identical to those determined for the WT β_1 and β_2ARs (Table 6-2). Additionally, the calculated K_i values for either carbostyril **1** or ISO at each of the TM IV chimeras constructed from the β_2AR were not significantly different from calculated K_i values for either carbostyril **1** or ISO at the WT β_2AR (Table 6-2 and Figure 6-3). Similarly, the TM IV chimera that was constructed from the β_1AR demonstrated affinities for carbostyril **1** and ISO similar to those calculated for the WT β_1AR. The apparent dissociation rate constants for carbostyril **1** and ISO from the full TM IV chimera [β_2S(149-173)] are identical to those determined at the WT β_1AR (Table 6-1 and Figure 6-6). The proximal 2/3 TM IV chimera [β_2S(149-166)] had a measurable decay in the steady state accumulation of cAMP after the addition of propranolol to whole cells pretreated with

carbostyril **1**, with a calculated apparent dissociation rate constant of 0.5 ± 0.1 min^{-1}. No measurable decay in the steady state accumulation of cAMP was observed after the addition of propranolol to carbostyril **1**-stimulated cells expressing either the distal 1/3 TM IV or the proximal 1/3 TM IV chimera, β_2S(164-173) and β_2S(149-158), respectively. Additionally, the calculated dissociation rate constant of carbostyril **1** from the β_1AR proximal 1/3 TM IV chimera, β_1S(177-181), which is the inverse of β_2S(149-158), was identical to the calculated apparent dissociation rate of carbostyril **1** from the WT β_1AR (Table 6-1 and Figure 6-6).

Discussion

Is there a common mechanism for structurally distinct agonists that enhance duration of action at the β_2AR? We believe an answer to this question depends upon the detailed analysis of multiple such agonists. In terms of its duration of action, the agonist behavior of carbostyril **1** at the β_2AR is similar to that of salmeterol, suggesting that carbostyril **1** may also interact with an exosite on this GCR. On the other hand, the inability of the antagonist propranolol to affect β_2AR activation by carbostyril **1**, at least within the time employed in our functional assays, is a striking difference in the pharmacology of these two agonists. This difference is further emphasized by the fact that carbostyril **1** is sensitive to antagonist in an identical manner to ISO at the β_1AR (Figure 3).

To probe the molecular basis for the differential response of carbostyril **1** to exogenous antagonist at the WT β_1AR and β_2AR, we characterized the apparent dissociation properties of carbostyril **1** at these two adrenergic receptors and a series of

chimeric β_1/β_2ARs. The use of chimeric GCRs to probe structural and functional aspects of subtype-selective, ligand recognition is well established, and has been employed to define the molecular basis of norepinephrine selectivity (Frielle *et al.*, 1988) and the salmeterol exosite (Green *et al.*, 1996). We have used these chimeric constructs to elucidate the structural requirements of the slow dissociation of carbostyril **1** from the β_2AR. The dramatic difference in the dissociation behavior of carbostyril **1** at β_1/β_2AR chimeras containing the β_2AR TM IV compared to those containing the β_1AR TM IV is striking. Furthermore, the fact the region of TM IV that confers salmeterol exosite binding, $Lys^{4.41(149)}$-$Trp^{4.50(158)}$, does not modulate the antagonist insensitive binding of carbostyril **1** implies two possibilities regarding the mechanism of slow dissociation from the β_2AR. The first possibility is that the receptor contains two exosites which form specific interactions with the side chain of either carbostyril **1** or salmeterol but not both. The salmeterol exosite ($Lys^{4.41(149)}$-$Trp^{4.50(158)}$) is distal from the active site which allows a remote tethering to allow for movement of the catecholamine pharmacophore away from the agonist-binding pocket as shown by salmeterol's antagonist sensitivity. The second exosite ($Ile^{4.51(159)}$-$Thr^{4.56(164)}$) specifically interacts with the side chain of carbostyril **1** to tether this ligand proximal to the agonist-binding site in such a way to not allow gross movements of the carbostyril nucleus away from the site responsible for agonist efficacy. This results in the apparent antagonist insensitivity of carbostyril **1** binding. Molecular modeling of the complex between salmeterol and the TM domains of the β_2AR have suggested that specific interactions exist between the side chain of salmeterol and

residues $Lys^{4.41(149)}$ to $Trp^{4.50(158)}$ of the β_2AR to support this hypothesis (Green *et al.*, 1996).

The second hypothesis may be that differences in the amino acid sequence of TM IV indirectly affect the conformational dynamics of the ligand/receptor complex (Gether *et al.*, 1997a; Gether *et al.*, 1997b). This hypothesis is consistent with previous reports suggesting that TM IV plays a critical role in β_2AR activation. First, a naturally occurring human polymorphism in which $Thr^{4.56(164)}$ is replaced by an isoleucine residue reduces the ability of the $T^{4.56(164)}I$ β_2AR mutant to respond to stimulation by β-agonists without affecting the agonist binding properties of the receptor (Green *et al.*, 1993). Second, the pharmacological characterization of a β_2AR mutant in which $Ser^{4.57(165)}$ is replaced by alanine also suggests that TM IV is involved in receptor signaling and activation (Nowicki *et al.*, 1997). This reduction in the agonist efficacy was due to a reduced ability of the receptor to form a ternary complex without affecting the agonist binding properties. Third, molecular dynamics simulations of the movements of helices with the receptor alone or with a full agonist, a partial agonist or an antagonist bound has proposed a model of the sequential dynamic events preceding receptor and G-protein activation. (Luo *et al.*, 1994). This simulation has strongly implicated TM IV as one of the initiators for helical movements that may change the conformation of both the third intracellular loop and the C-termini which could effect G-protein coupling. These simulations have gained experimental support with recent work to investigate the ability of agonists, antagonists and inverse agonists to change the quenching properties of a florescently labeled receptor (Gether *et al.*, 1995). The degree of fluorescent quenching following the binding of

various ligands correlated with the efficacy of each ligand. This suggested to the authors that each ligand caused varying degrees of dynamic movements in the receptor based upon the efficacy of the ligand bound. These results were very similar to those obtained independently using molecular dynamic simulations. Therefore, it is possible that the non-conserved residues interact with other residues in the receptor to change the local microenvironments in which the side chain of these agonists is bound. These local changes in the dynamics of the receptor may be enough to affect the dissociation kinetics of each of these novel β-agonists. Additionally, the antagonist sensitivity may be just a function of side chain length. There may be a greater ability of the pharmacophore to move in and out of the agonist-binding pocket with a longer side chain. Therefore, it is the challenge for future experiments to determine the mechanism of action involved in the slow dissociation of the novel β-agonists and to determine if these two hypotheses constitute a common or divergent mechanism of action with either the carbostyril **1** or salmeterol.

Another hypothesis that has been considered in Chapter 5 is that the dissociation rate of the carbostyril agonists is dependent solely on the affinity of the agonists. The k_{off} is a function of both the K_D and the k_{on}. Assuming a constant value for k_{on} as suggested in chapter 5, a ligand with a very high affinity will dissociate very slowly from the receptor. For this hypothesis to be valid, any mutation that affects the dissociation kinetics will affect the affinity of the ligand for that receptor. The data obtained using the chimeric receptors are not consistent with this hypothesis. Several examples of receptors with low affinity for carbostyril **1** retain slow-dissociation kinetics for the ligand. β_2(I-

VI)/β_1(VII)AR has an affinity comparable to the WT β_1AR however, carbostyril **1** slowly dissociates from this receptor in a manner identical to the WT β_2AR. Conversely, the β_2AR TM IV chimeras have identical affinities for carbostyril **1** that are similar to the WT β_2AR; however, only two of the receptors have dissociation kinetics similar to the WT β_2AR. These results indicate that the dissociation rate and the affinity of carbostyril **1** can be dissociated. Additionally, the structural determinates for high affinity binding and slow-dissociation kinetics may be entirely different.

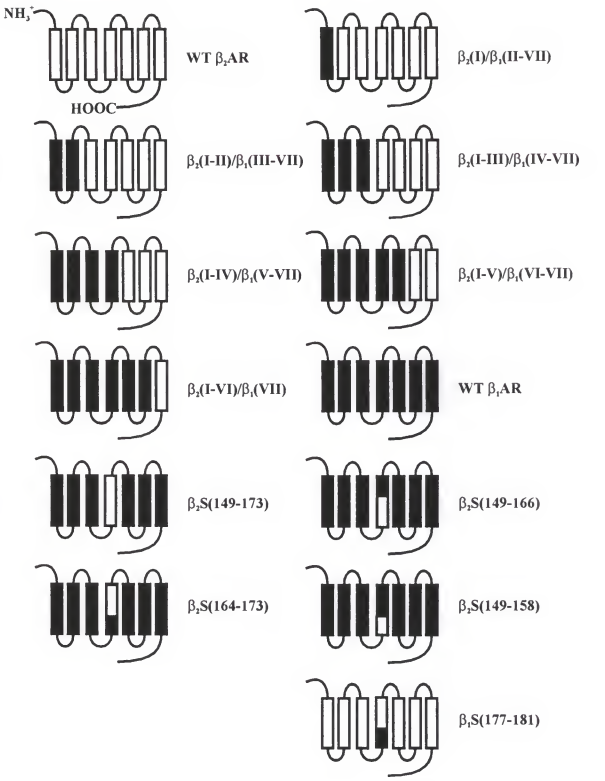


Figure 6-1. **Schematic representation of the chimeric receptors tested.** All β_1AR and β_2AR amino acid sequences in the TM domains are indicated by black and white rectangles, respectively.

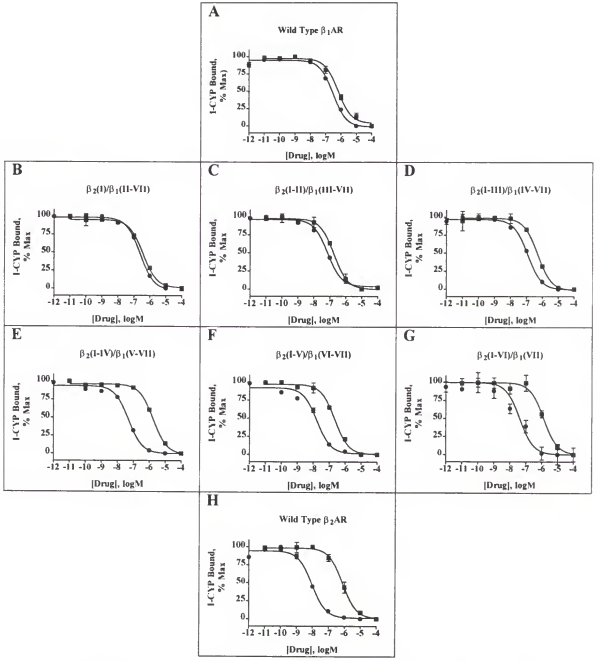


Figure 6-2. [^{125}I]-CYP **radioligand competition data for carbostyril 1 and ISO using murine L cells expressing WT β_1 and β_2ARs, and β_2 / β_1AR chimeras.** Data are shown as the mean ± S.E.M. for three or four experiments; see also Table 6-1. Murine L cells were stably transfected with the appropriate plasmid constructs, membranes were prepared, and competition binding assays were performed in the presence of 100 μM Gpp(NH)p and the indicated concentrations of carbostyril **1** (●) and ISO (■), as described in Chapter 2. Mean data shown in Table 6-2.

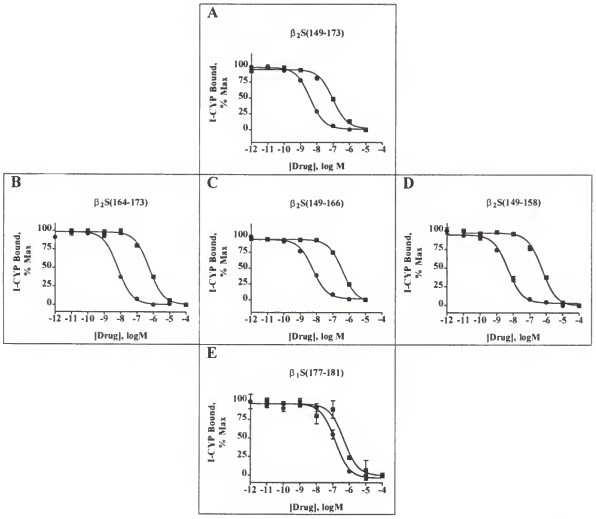


Figure 6-3. [^{125}I]-CYP **radioligand competition data for carbostyril 1 and ISO using murine L cells expressing β_2 / β_1AR TM IV chimeras.** Murine L cells were stably transfected with constructs encoding β_2S(149-173) (**A**),β_2S(149-166) (**B**),β_2S(164-173) (**C**),β_2S(149-158) (**D**), and β_1S(177-181) (**E**) ARs, membranes were prepared, and competition binding assays were performed in the presence of 100 μM Gpp(NH)p and the indicated concentrations of carbostyril **1** (●) and ISO (■), as described in "Chapter 2". Data are shown as the mean ± S.E.M. for three or four experiments; see also Table 6-1. Mean data shown in Table 6-2.

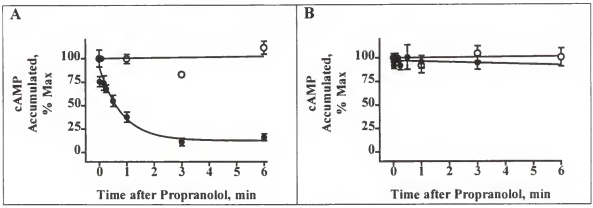


Figure 6-4. **Apparent dissociation behavior of carbostyril 1 at the WT β_1AR and the WT β_2AR.** Murine L cells were stably transfected with constructs encoding either the WT β_1AR (Panel A) or the WT β_2AR (Panel B). Whole cells were isolated and pre-treated at 37 ^{O}C with 1 μM carbostyril **1** (circles) for 4 min. The amount of cAMP accumulated was monitored after the addition of propranolol (20 μM) (filled symbols) or buffer (open symbols), as described in Chapter 2. Results are presented as the fraction of maximal cAMP accumulated for each L cell clone and are the mean ± S.E.M. for three experiments; see also Table 6-1.

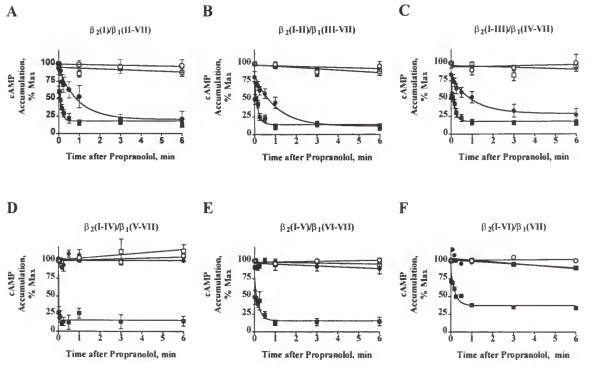


Figure 6-5. **Apparent dissociation behavior of carbostyril 1 and ISO at β_1/β_2AR chimeras.** Murine L cells were stably transfected with constructs encoding β_2(I)/β_1(II-VII) (**A**), β_2(I-II)/β_1(III-VII) (**B**), β_2(I-III)/β_1(IV-VII) (**C**), β_2(I-IV)/β_1(V-VII) (**D**), β_2(I-V)/β_1(VI-VII) (**E**) and β_2(I-VI)/β_1(VII) (**F**) chimeric ARs. Whole cells were isolated and pre-treated at 37 ^{o}C with either 1 μM carbostyril **1** (circles) or ISO (squares) for 4 min. The amount of cAMP accumulated was monitored after the addition of either 20 μM propranolol (filled symbols) or buffer (open symbols), and the decrease in agonist-stimulated adenylyl cyclase activity determined, as described in Chapter 2. Results are presented as the fraction of maximal cAMP accumulated for each chimeric β_1/β_2AR, and are the mean ± S.E.M. for three experiments; see also Table 6-1.

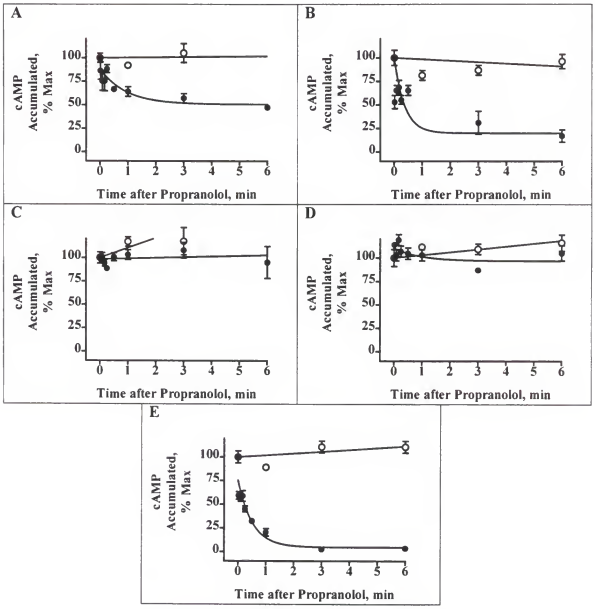


Figure 6-6. **Apparent dissociation behavior of carbostyril 1 at β_1 and β_2AR TM IV chimeras.** Murine L cells were stably transfected with constructs encoding β_2S(149-173)AR (Panel **A**),β_2S(149-166)AR (Panel **B**),β_2S(164-173)AR (Panel **C**),β_2S(149-158)AR (Panel **D**), and β_1S(177-181)AR (Panel **E**), whole cells isolated and pre-treated at 37 ^{o}C with 1 μM carbostyril **1** (circles) for 4 min. The amount of cAMP accumulated was monitored after the addition of either 20 μM propranolol (filled circles) or buffer (open circles) and the decrease in agonist-stimulated adenylyl cyclase activity for carbostyril **1** determined, as described in "Chapter 2". Results are presented as the fraction of maximal cAMP accumulated for each $\beta_1$or β_2AR TM IV mutant, and are the mean ± S.E.M. for three experiments; see also Table 6-1.

CHAPTER 7
MOLECULAR MODELING OF THE COMPLEX FORMED BETWEEN THE CARBOSTYRIL-BASED AGONIST **1** AND THE HUMAN β_2-ADRENERGIC RECEPTOR

Introduction

As recently discussed (Ballesteros and Weinstein, 1995), simple minimization and comparison of relative energies for various ligand/receptor complexes is not sufficiently stringent for the interpretation of structure-activity relationships. Indeed, the structural requirements for ligand binding can probably be fulfilled by different combinations of side chains from residues in different TM domains of the receptor model (Ballesteros and Weinstein, 1995). Such simulations are easily carried out using well-established molecular dynamic (MD) techniques (Brooks *et al.*, 1988). This approach to delineating receptor/ligand interactions is reliable in understanding the effects of site-specific mutations given that subtle structural reorganization might occur at mutated sites compensating for the modifications to the protein introduced by the mutation. In addition, such studies on a model of the $5HT_2$ subtype of the serotonin receptor reveal characteristic series of structural changes produced in complexes involving agonists (Zhang and Weinstein, 1993). These motions can be used to validate models given that their presence should correlate with agonist activity, i.e. they should be diminished in complexes of weak, or partial, agonists and absent in the case of antagonist/receptor models. With the data necessary to accurately describe the binding site of the carbostyril

nucleus within the transmembrane segments of the β_2AR is available, a model of the complex between carbostyril 1 and the β_2AR can be constructed.

Methods

Homology modeling of the β_2AR based on the atomic coordinates of rhodopsin was greatly expedited with the use of the Swiss-Model 7TM Interface at http://www.expasy.ch/cgi-bin/sm-gpcr.pl. The Swiss-Model 7TM Interface was used to generate an initial structure based on the helical packing of bovine rhodopsin (Herzyk and Hubbard, 1995). The amino acid sequences for each of the TM domains used for the Swiss model automated sequence are depicted in Table 7-1.

Table 7-1.
Amino acid sequence of the TM domains used in the Swiss Protein automated model builder.

TM Domain	Amino Acid Sequence
I	V G M G I V M S L I V L A I V F G N V L V I T A I A
II	Y F I T S L A C A D L V M G L A V V P F G A A H I I
III	E F W T S I D V L C V T A S I E T L C V I A V D R Y
IV	K A R V I I L M V W I V S G L T S F L P I Q M H W Y
V	A Y A I A S S I V S F Y V P L V I M V F V Y S R V F
VI	K A L K I L G I I M G T F I L C W L P F F I V N I V
VII	L N W I G Y V N S G F N P L I Y C R S P D F R I A F

This initial model contained the interhelical interactions as well as having the essential ligand binding residues in the proper orientation for ligand coordination (Table 7-2). The TM domains were edited using MacroModel V2.0 (Mohamadi *et al.*, 1990) to achieve the proper TM domain lengths according to Donnelly *et al.* (1994).

Table 7-2.

The important inter- and intra-helical contact points maintained during construction of starting structures.

Interaction (constrained distance in Å)	Residue 1	Residue 2
H-Bond (~1.8)	$Asn^{2.50(79)}$	$Asp^{7.49(322)}$
Electrostatic (~2.5)	$Arg^{3.50(131)}$	$Asn^{2.50(79)}$
α-Helical Backbone H-Bond (~1.8)	Backbone Amide Proton Residue n	Backbone Carbonyl Oxygen Residue n+4
Electrostatic (~2.5)	$Asp^{3.32(113)}$	Protonated Amine of Carbostyril **1**
H-bond (~1.8)	$Ser^{5.46(207)}$	8-Hydroxy Moiety of Carbostyril **1**
H-bond (~1.8)	$Ser^{5.43(204)}$	Amide Carbonyl Moiety of Carbostyril **1**
H-bond (~1.8)	$Asn^{6.55(293)}$	β-Hydroxy Moiety of Carbostyril **1**

The amino acid sequence of each of the TM domains in the final starting structure is depicted in Table 7-3.

Table 7-3.

Amino acid sequence of the TM domains used in the final β_2AR model.

TM Domain	Amino Acid Sequence
I	EVWVVGMGIVMSLIVLAIVFGNVLVITAIAKFERL
II	VTNYFITSLACADLVMGLAVVPFG
III	FWCEFWTSIDVLCVTASIETLCVIAVDRYFAITS
IV	TKNKARVIILMVWIVSGLTSFLPIQM
V	NQAYAIASSIVSFYVPLVIMVFVYSRVFQEAKR
VI	KEHKALKTLGIIMGTFTLCWLPFFIVNIVHVIQ
VII	IRKEVYILLNWIGYVNSGFNPLIYCRSPDFRIAF

Each helix was graphically capped by acetamide at its N-terminus and *N*-methylamide at the C-terminus. These structural modifications were made using MacroModel V2.0. Any errors in either residue connectivity or residue identification were corrected using the Florida Interactive Molecular Modeling System (FLIMMSY). Carbostyril **1** was built and

docked into the binding site of the model using MacroModel V2.0. Sequential energy minimization calculations were carried out using BATCHMIN V5.0 with progressively decreasing constraints on the backbone α-helical hydrogen bonding, on the interhelical contact points, and the receptor ligand interactions (Table 7-2) until a final gradient of 0.01 Kcal/mole/Å was obtained without the inclusion of energy constraints. The minimization procedure consisted of a minimum number of steps of Polak-Ribiere conjugate gradient minimization using BATCHMIN V5.0 with the Amber* set of parameters (McDonald and Still, 1992). The Amber* parameters utilize the current 6,12-Lennard Jones Treatment (Weiner and Kollman, 1986) Extended nonbonding interactions parameters were used and are specifically set at 8 Å for nonbonded interactions, 4 Å for H-bonding interactions and 20 Å for charge/charge electrostatic interactions to obtain a final gradient of 0.01 kcal/mole/Å. The resultant structure constitutes the starting structure for molecular dynamics (MD) simulations. MD simulations were carried out using BATCHMIN V5.0 (Mohamadi *et al.*, 1990) after 10 initial 20 pS equilibration steps with progressively decreasing constraints. The MD simulations were carried out with the following conditions: cutoffs 8 Å for nonbonded interactions, 4 Å for hydrogen bonds and 20 Å for charge/charge electrostatics; step size of 1.5 fs; and constraints on bonds with hydrogens by the SHAKE alogorithm (van Gunsteren and Berendsen, 1977). The simulation was carried out at a constant temperature of 300 K using a thermal bath (Berendsen *et al.*, 1984) with a bath time constant of 0.2 pS. The final unconstrained dynamics simulation was for 250 pS at 300 K. Structures were sampled every 1 pS of simulation. Each structure was minimized and all unique structures were saved. The

unique structures were inspected for the maintenance of critical interactions between the helices and between the receptor and the ligand. The lowest energy structure consistent with all experimental data was chosen as the final structure.

All coordinate manipulations and energy calculations were done using MacroModel V2.0. Energy minimization of the initial complex was carried out using the AMBER* parameters and potential energy functions as implemented in BATCHMIN V5.0.

Results

The construction of a structural model of the complex between carbostyril ligand **1** and the TM domains of the β_2AR has been performed to test the hypothesis that a discrete exosite in TM IV mediates the slow dissociation kinetics observed for carbostyril **1** by binding the ligand side chain. Homology modeling of GPCRs has recently gained great application in the rationalization of site-directed mutagenesis studies using MD simulations (Ballesteros and Weinstein, 1995). The use of MD simulations have also been used extensively in the study of ligand induced conformational changes in the family of GPCRs (Luo *et al.*, 1994; Strahs and Weinstein, 1997). MD simulations was used in the construction the model of the complex between carbostyril **1** and the β_2AR in order to insure to some degree that what was generated in terms of energy was not a single energy minimum completely dependent upon the conformation of the starting structure. It is the ability of MD to randomly change the conformation of the structure to allow an adequate examination of the possible conformations available that result in the identification of energy minimums that are distant from the starting structure.

The quality of the final structure is dependent in large part on the quality of the starting structure. Therefore, a large amount of experimental data was taken into account when constructing the initial model of the complex between carbostyril **1** and the β_2AR. The position of the α-carbons is a most important aspect of the model and represents the template for model building. The template chosen used the α-carbon coordinates provided through the Swiss-model 7TM interface. This template is based upon the published electron cryomicroscopy data for bovine rhodopsin (Schertler *et al.*, 1993). The interface allows the input of amino acid sequence for each TM segment with a required TM segment length of 26 amino acids (Table 7-1). The Swiss-Model 7TM Interface places each amino acid side chain on the appropriate α-carbon and minimizes the structure a few steps to eliminate obvious Van der Waal and electrostatic conflicts. The structure was inspected to insure all structural requirements could be accommodated (Table 7-2). The length of each TM domain was altered using MacroModel V2.0 to be the proper length according to the TM domain boundaries described by Donnelly *et al.* (1994). The N-terminal and C-terminal ends of each helix was capped with acetamide and *N*-methylamide, respectively.

Carbostyril **1** was built and subsequently docked within the agonist-binding site of the β_2AR graphically using MacroModel V2.0. The carbostyril pharmacophore was placed so that hydrogen bonds formed between the side chains of $Ser^{5.46(207)}$ and $Asn^{6.55(293)}$, and the 8-hydroxy substituent and the β-hydroxyl group, respectively. Additionally, the side chain was positioned to allow an electrostatic interaction between $Asp^{3.32(113)}$ and the protonated amine on the side chain of carbostyril **1**. The remainder of the side chain was

placed within the agonist-binding site and all unwanted steric interactions were eliminated graphically. This initial model of the complex was subjected to energy minimization with constraints on the distances between the ligand functional groups and the coordinating side chains within the agonist-binding site, between the amide protons and amide carbonyls that form the hydrogen bonding network that stabilizes the α-helical secondary structure, and between the functional groups of the residues involved in interhelical interactions. Several energy minimization protocols were performed with progressively smaller constraints until the complex was minimized without constraints to a final gradient of 0.01 kcal/mole/Å.

MD simulations were initialized by equilibrating the structure in 10 pS time steps with progressively decreasing constraints on the important distances listed in Table 7-2. The final MD simulation consisted of 250 pS of MD without constraints. Structures were sampled every picosecond during the simulation, minimized and all unique structures were saved. The final structure consisted of the lowest energy structure that maintained all of the important interactions that have been confirmed experimentally.

The final structure (Figure 7-1) maintained a helical packing arrangement similar to that reported for bovine rhodopsin (Schertler *et al.*, 1993). All interactions between the carbostyril pharmacophore and the agonist-binding site were maintained in the final structure (Figure 7-2). Hydrogen bonding interactions between $Asn^{6.55(293)}$ and the β-hydroxyl substituent of the ligand, $Ser^{5.46(207)}$ and the *para*-hydroxyl substituent, and electrostatic interactions between the charged amino function of the ligand and the carboxylate function of the side chain of $Asp^{3.32(113)}$. For ISO, the $Ser^{5.43(204)}$ hydroxyl

group is hydrogen bonded with the *meta*-catechol hydroxyl. However, hydrogen bonding interactions with $Ser^{5.43(204)}$ and the amide carbonyl of the carbostyril nucleus is not maintained with carbostyril **1** bound within the agonist-binding site. This is consistent with the ambiguous role of $Ser^{5.43(204)}$ in the coordination of the carbostyril nucleus based on the complementary mutagenesis data. Additionally, all backbone hydrogen bonding interactions remained intact. There were no gross alterations in the α-helical structure of the TM domains. Finally, the interhelical contact points that determine the relative orientation of TM II, III, and VII were maintained (Figure 7-3). The interaction of $Arg^{3.50(131)}$ with $Asp^{2.50(79)}$ and not $Asp^{3.49(130)}$ indicates that the conformation studied may represents the active conformation of the β_2AR.

The side chain of carbostyril **1** appeared to be interacting with residues located at the interface of TM II and VII. However, no specific interactions were observed with residues located in TM IV. The region of TM IV that was shown experimentally to confer slow dissociation of carbostyril **1** from the β_2AR does not directly interact with the side chain of carbostyril **1** (Figure 7-4). Differences in the amino acid sequence of TM IV between the β_1 and β_2ARs are located at interhelical interfaces of TM IV with TM III and V. Specifically, $Gly^{4.54(162)}$ is an alanine in the β_1AR which is located in a position to interact with several amino acids in TM III. Additionally, $Thr^{4.56(164)}$ is a valine in the β_1AR which may directly change the interaction with $Tyr^{5.38(199)}$.

Discussion

Using similar modeling approaches to those reported previously (Ballesteros and Weinstein, 1995; Gouldson *et al.*, 1997; Donnelly *et al.*, 1994; Maloney-Huss and

Lybrand, 1992; Kontoyianni *et al.*, 1996) and employing a template based on the helix-packing observed for bovine rhodopsin (Schertler *et al.*, 1993), we have constructed a preliminary model of the complex between carbostyril **1** and the seven TM helices of the β_2AR (Figure 7-1). This model suggests that when the carbostyril moiety of carbostyril **1** is bound in the catecholamine-binding site, the β-agonist side chain form a series of interactions with residues in the interhelical interface of TM II and VII. These interactions however, are not specific to any non-conserved residues in either of these TM domains. This is consistent with experimental data presented in Chapter 6 that indicates that neither TM II nor VII mediate the slow dissociation kinetics observed for carbostyril **1** from the β_2AR. Additionally, there are no direct interactions between carbostyril **1** and the fourth TM domain. Therefore, the results of this structural model do not support the hypothesis that direct, static interactions tether the side chain of carbostyril **1** to achieve its antagonist insensitive, extended duration of action. The model is consistent however, with the findings that TM VII modulates the high-affinity binding of TA-2005 (carbostyril **9**). Kikawa *et al.* (1998) identified $Tyr^{7.35(308)}$ as the important residue responsible for high-affinity binding of carbostyril **9**. Although, TM VII did not modulate the high-affinity binding of carbostyril **1** (Chapter 6, Table 6-2) it does not rule out the possibility that the side-chain of carbostyril **1** is interacting with this region of TM VII.

The second hypothesis concerning the mechanism of the slow dissociation of carbostyril **1** from the β_2AR is that TM IV modulates the dynamics of the receptor. These amino acid differences between $Ile^{4.51(159)}$ to $Thr^{4.56(164)}$ in TM IV may allow conformational changes in the β_2AR that are responsible for the pseudo-irreversible

binding of carbostyril **1** to an adjacent binding site. The amino acid composition of the adjacent binding site may be identical in both of the subtype; however, the dynamics of the binding site may be dramatically different. The identification of non-conserved residues at the interhelical junction of TM IV with TM III and V suggests that this hypothesis may be correct. These TM domains have been shown important for both ligand binding and/or receptor activation. TM III and V are important for the specific binding of agonists through the electrostatic interactions with $Asp^{3.32(113)}$ and the hydrogen bonding interactions with $Ser^{5.46(207)}$ and/or $Ser^{5.43(204)}$, respectively (Strader *et al.*, 1994). Additionally, TM IV has been shown to be important in modulating the activity of the receptor without being directly involved in agonist binding (Green *et al.*, 1993; Nowicki *et al.*, 1997). In this model, complex interactions between residues in TM IV and those residues in TM III and V are proposed that may effect the interaction of these helices with the carbostyril pharmacophore. However, nothing is known regarding the helical motions during the 250 pS of MD simulation because no considerations of the movements were made. The structure shown represents the lowest energy structure from the 250 sampled during the MD simulation.

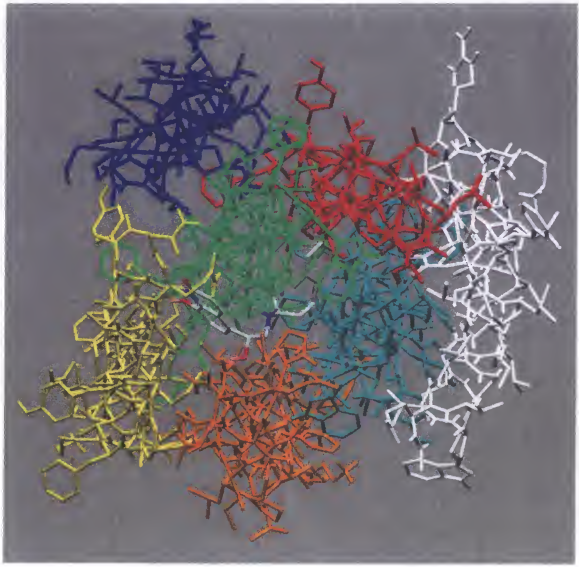


Figure 7-1. **The model of the complex between the β_2AR and carbostyril 1.** The model of the complex between the β_2AR and carbostyril **1** is viewed from the extracellular face of the protein. The TM domains are present as described in Table 7-3. Each TM domain is color coded. TM I is white, TM II is red, TM III is green, TM IV is blue, TM V is yellow, TM VI is orange, TM VII is Turquoise and carbostyril **1** is colored by atom type. The model was generated as described in the Methods section of Chapter 7 and all color coding is maintained throught the figures.

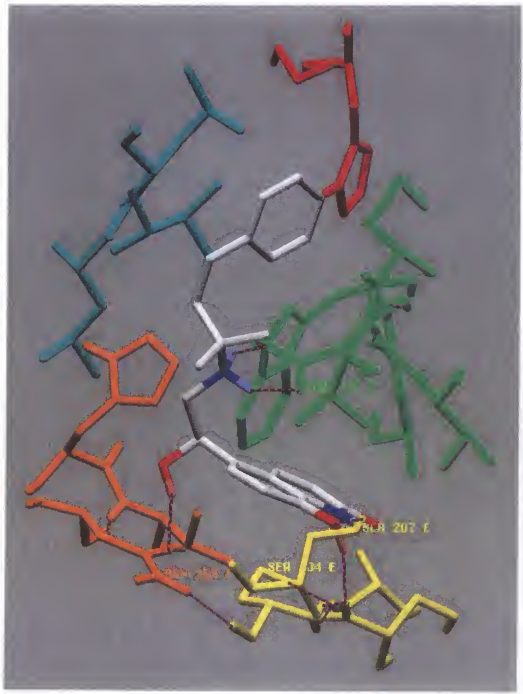


Figure 7-2. **Carbostyril 1 bound within the agonist-binding site of the β_2AR.** This view illustrates the important interactions between the residues in the agonist-binding site and the functional groups of carbostyril **1**. Pink dotted lines indicate hydrogen bonding interactions.

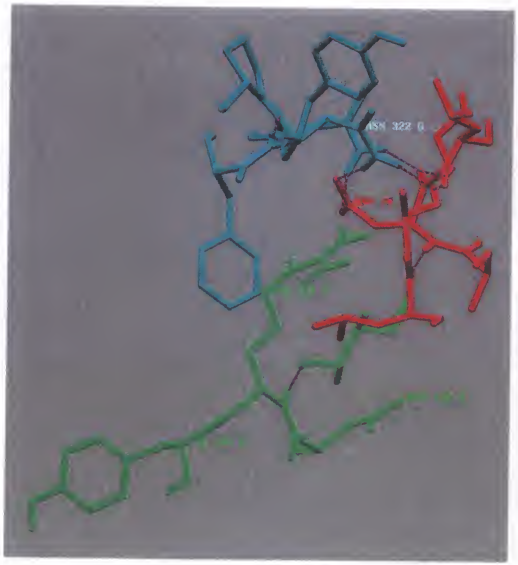


Figure 7-3. **The interaction of $Arg^{3.50(131)}$ with the conserved interhelical hydrogen bond between $Asp^{2.50(79)}$ and $Asn^{7.49(322)}$.** This view of the model of the complex between carbostyril **1** and the β_2AR illustrates that the model includes many structural features that insure the proper orientation of many of the TM domains. The interaction of $Arg^{3.50(131)}$ with $Asp^{2.50(79)}$ and not $Asp^{3.49(130)}$ indicates that the conformation studied represents the active conformation of the β_2AR.

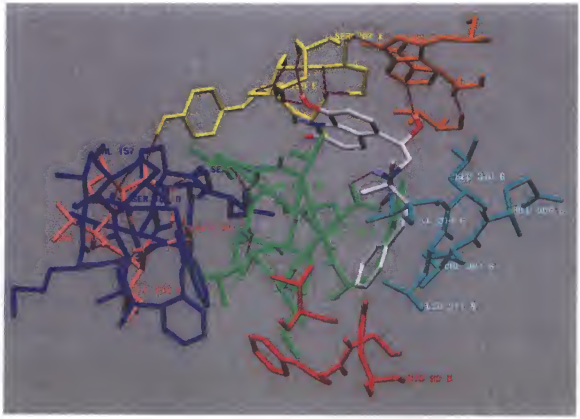


Figure 7-4. **Interaction of carbostyril 1 with the agonist binding site and adjacent non-conserved structures in TM IV.** This view of the complex between the β_2AR and carbostyril **1** shows the important amino acids that coordinate the interaction of the carbostyril nucleus within the agonist-binding site. Additionally, the portion of the TM IV domain that confers slow dissociation behavior of carbostyril **1** is shown (blue) with the non-conserved residues indicated in pink.

CHAPTER 8
CONCLUSIONS AND FUTURE DIRECTIONS

Recently a novel class of carbostyril-based βAR agonists was developed in Dr. Baker's laboratory. These compounds have been shown to exhibit slow dissociation characteristics that are selective for the β_2AR. These compounds rapidly dissociate from the β_1AR. This selectivity offers an additional theoretical advantage of reduced side effects resulting from possible long term over stimulation of the β_1AR. This novel class of βAR agonists appears to have this property due to specific interactions within the ligand-binding site of the β_2AR. The novel carbostyril compounds may have value as therapeutic agents for the treatment of cardiovascular disease and to reduce the cardiovascular side effects often associated with more traditional agonist for the treatment of asthma. The unique properties of the carbostyril derivatives have allowed us to implement an interactive multidisciplinary experimental approach (pharmacology, molecular biology, and molecular modeling) to investigate some fundamental issues concerning slow dissociating βAR agonists.

The first objective was to determine the role of a conserved serine in the fourth TM domain of the β_2AR. Previously, $Ser^{4.56(165)}$ was implicated, in separate molecular modeling studies, in forming a hydrogen bond interaction with the β-hydroxyl moiety of the catecholamine side chain. However, initial attempts to generate the mutation failed due to the inability to express the hamster $S^{4.56(165)}$A mutant β_2AR. Recently, Weiland *et al.* (1996) published binding data using a human $S^{4.56(165)}$A mutant β_2AR that indicated

that $Ser^{4.56(165)}$ was not involved in ligand binding. Subsequently, our laboratory generated the human $S^{4.56(165)}A$ mutant β_2AR to determine the role of conserved amino acid in the function of the β_2AR and confirmed that this residue is not directly involved in ligand coordination. This particular residue appears to be involved in the signal transduction of the β_2AR by affecting the amount of ternary complex formed between the receptor, agonist and G-protein. A reduction in ternary complex formation of the human $S^{4.56(165)}A$ mutant β_2AR was indicated by the inability of GTP analogs to right-shift the agonist competition curves and in the reduction in the NEM sensitivity of radioligand binding of the receptor as compared to WT β_2AR. The consequence of this mutation was noticeable when stimulating the human $S^{4.56(165)}A$ mutant β_2AR with the partial agonist dobutamine. The human $S^{4.56(165)}A$ mutant β_2AR had a reduced response to dobutamine when compared to the WT β_2AR. However, full agonists retained full efficacy at both the WT and $S^{4.56(165)}A$ mutant β_2AR. This data is consistent with previously published radioligand and functional data using the human $T^{4.55(164)}I$ mutant β_2AR. This mutant receptor had an impaired ability to stimulate the accumulation of cAMP with full agonists, and agonist competition curves were insensitive to the addition of GTP analogs as compared to the WT β_2AR (Green *et al.*, 1993). Therefore, TM IV appears to indirectly modulate the activity of the receptor by possibly affecting the dynamic interactions between TM domains III and V.

The next step in our understanding of the interaction of carbostyril **1** with the β_2AR involved the elucidation of the agonist-binding site for the carbostyril nucleus. The hypothesis was that the catecholamine-binding site that was identified previously using site-directed mutagenesis served as the carbostyril-binding site. Therefore,

oligonucleotide directed site specific mutagenesis of the β_2AR was employed to determine the structural and/or functional roles of residues located within the TM domains that contribute to carbostyril binding. Specifically, $Ser^{5.46(207)}$ and $Ser^{5.43(204)}$ have been shown to interact with the *para*- and *meta*- hydroxyl moieties of catecholamine agonists, respectively, and have been mutated to alanine. $Asp^{3.32(113)}$ has been demonstrated to form an electrostatic interaction with the protonated amine of the side chain of catecholamine ligands and was mutated to a glutamate residue.

In our studies, the genes encoding either the WT or the mutant human β_2ARs were successfully transfected into mouse L cells. Clonal cells were isolated which expressed similar levels of receptor. All human β_2AR mutants showed identical affinity for the radiolabeled antagonist, [^{125}I]-CYP indicating that the receptors were properly folded within the membrane. In competition experiments of [^{125}I]-CYP binding, increases in the K_I for ISO of approximately 21- and 17-fold were observed with the $S^{5.43(204)}$A and $S^{5.46(207)}$A mutants, respectively. These results are very similar to those previously reported for the cognate mutations in the hamster β_2AR. On the other hand, similar experiments using carbostyril **1** revealed subtle differences in its interactions with the catecholamine-binding site of the β_2AR. Increases in the carbostyril **1** K_I of approximately 9.6- and 3.9-fold were observed, however, with the $S^{5.43(204)}$A and $S^{5.46(207)}$A human β_2AR mutants, respectively.

To determine the orientation of the functional groups on the carbostyril nucleus with respect to $Ser^{5.43(204)}$ and $Ser^{5.46(207)}$, an approach was taken to introduce complementary changes to the ligand that will restore binding affinity at either the $S^{5.43(204)}$A and $S^{5.46(207)}$A human β_2AR mutants. Complementary changes to the ligand

that were introduced were designed to allow a hydrophobic interaction instead of the wild type hydrogen bonding interactions. Therefore, two methylated derivatives of carbostyril **1** were synthesized. Carbostyril **7** and **8** exchanged either the 8-hydroxy proton or the amide proton with a methyl group, respectively. Carbostyril **7** was shown to have a lower affinity at both the WT and $S^{5.43(204)}$A mutant β_2ARs as compared to carbostyril **1**. This is consistent with a single and a double loss of hydrogen bond coordination, respectively. However, the affinity of carbostyril **7** at the $S^{5.46(207)}$A β_2AR mutant was identical to the affinity of carbostyril **1** at the WT β_2AR. Additionally, the reduced efficacy of carbostyril **7** observed at the WT and $S^{5.43(204)}$A mutant β_2ARs was increased at the $S^{5.46(207)}$A β_2AR mutant. These results provide convincing evidence for a hydrophobic interaction between the $Ala^{5.46(207)}$ and the 8-methoxy function of the carbostyril nucleus. Similarly, carbostyril **8** was shown to have a lower affinity at the WT, $S^{5.43(204)}$A mutant, and $S^{5.46(207)}$A mutant β_2ARs as compared to carbostyril **1**. However, the rank order in the affinity was changed in that the affinity of carbostyril **8** at the $S^{5.46(207)}$A mutant β_2AR was higher than at both the WT and $S^{5.43(204)}$A mutant β_2ARs. This data is consistent with the possibility that the amide proton forms a hydrogen bond with $Ser^{5.43(204)}$; however, the affinity was still reduced compared to the affinity of carbostyril **1** at the WT β_2AR. Unexpectedly, carbostyril **8** was an antagonist at the WT, $S^{5.43(204)}$A mutant, and $S^{5.46(207)}$A mutants. Therefore, it could not be determined if complementation is possible between the methylamide function and $Ala^{5.43(204)}$. The side chain protonated amine of carbostyril **1** appears to form an electrostatic interaction with the carboxylate group of $Asp^{3.32(113)}$ because the affinity of carbostyril **1** at the $D^{3.32(113)}$E mutant β_2AR was

dramatically reduced compared to the WT β_2AR. Overall, this data suggest that the carbostyril-binding site is analogous to the catecholamine-binding site.

Another important aspect of our understanding of the mechanism of slow dissociation of carbostyril **1** from the β_2AR is to determine the structural features of carbostyril **1** important for slow dissociation. These structural features were determined by synthesizing several derivatives that alter different segments of the side chain phenteramine to assess the effect of these structural modifications on affinity for and slow dissociation from the β_2AR. In derivative **11**, the phenteramine in replaced with propylamine whereas compounds **12** and **6** lack only the geminal methyls or the phenyl ring, respectively. The number of methylene spacers between the carbon bearing the geminal methyl groups and the phenyl ring was changed to zero (**13**) and three (**14**). In compound **15,** the amino function carries an isopropyl moiety rendering the side chain analogous to ISO. Finally, in derivative **16**, catechol replaced the 8-hydroxycarbostyril nucleus of compound **1** to investigate if this ring contributes to slow dissociation.

It appears that the antagonist-insensitive, pseudo-irreversible binding of carbostyril **1** is due to a complex relationship between the structure of the side chain and the 8-hydroxycarbostyril nucleus. The important aspects of the structure of the side chain are the presence of geminal methyl groups, a vicinal phenyl function and the length of the linker between these two functional groups. This was demonstrated using both functional assays measuring the accumulation of cAMP and in binding assays that measured the residual inhibition of [^{125}I]-CYP binding. Both of these methods show that the action of carbostyril **1** is of long duration. There also appears to be one derivative that displays intermediate duration of action in both assays. In addition to the effect on the dissociation

rate, the structural alterations produced proportional changes in the affinity of each of the carbostyril derivatives for the β_2-AR. The data is suggestive of a thermodynamic model to explain the slow dissociation observed for carbostyril **1** at the β_2-AR. A thermodynamic model would be based on the relationship between the on and off rates of a ligand and the affinity. Therefore, the dissociation rate of each compound would simply be a function of the affinity and would be seen with all ligands having very high affinities.

Another important aspect of this study was to determine the specific transmembrane spanning region(s) of the β_2AR that confer the subtype selective slow dissociation characteristics of carbostyril **1**. It can be assumed that the amino acid sequence of the β_2AR contributes to the slow-dissociation of carbostyril **1** in that the ligand rapidly dissociates from the β_1AR. The differences are quite small in the core portion of the TM domains (42 differences out of 162 residues). The most differences are found in TM IV and VII. TM IV was found to be responsible for NE/Epi selectivity and TM VII was found to be responsible for antagonist selectivity using chimeric β_2/β_1ARs. The selectivity of carbostyril **1** was characterized in terms of the β_2AR TM domain required for the effect using chimeric β_2/β_1ARs. It was found that TM IV is required for the slow-dissociation of carbostyril **1** from the β_2AR. This result appears similar and consistent with the model of exosite binding to explain the slow-dissociation of salmeterol.

The exosite model of salmeterol binding was proposed as a result of experimental data that suggested that residues located in the intracellular 1/3 of TM IV of the β_2AR are responsible for slow-dissociation (Green *et al.*, 1996). Using identical constructs, the

region of TM IV responsible for carbostyril **1** slow dissociation is the middle 1/3 of this TM domain. The discrete region responsible for modulating the slow-dissociation of salmeterol has no effect on the dissociation of carbostyril **1**. In addition, the affinity of carbostyril **1** for each of the chimeric receptors did not correlate with the dissociation rate. This indicates there is dissociation between the structural requirements that determine the dissociation rate and the affinity of carbostyril **1**. Therefore, an isomerization event within the β_2AR may be responsible for the pseudo-irreversible binding of carbostyril **1**. This isomerization event may be mediated through interactions between non-conserved amino acids located in TM IV and adjacent regions of the β_2AR.

Molecular modeling studies were initiated to determine if it was possible for direct interactions between side chain of carbostyril **1** and the middle 1/3 of TM IV with the carbostyril pharmacophore coordinated within the agonist-binding site. This arrangement would suggest an additional exosite within the fourth TM domain that could modulate the dissociation behavior of carbostyril **1**. A model of the complex between carbostyril **1** and the TM domain of the human β_2AR was constructed using energy minimization techniques with limited molecular dynamic simulations. Although the model properly coordinated the carbostyril nucleus within the agonist binding site, the side chain appeared to make specific interactions with residues at the interface between TM domains II and VII. This data is consistent with TM VII having a role in the high affinity binding of carbostyril-based agonists. However, it is not consistent with the hypothesis that an alternative exosite exists for the side chain of carbostyril **1** to confer slow-dissociation at the β_2AR. Alternatively, the model is consistent with the hypothesis that there is dissociation between the structural requirements for high affinity binding and

slow dissociation rates. The structural determinates for high affinity binding appears to correlate with non-conserved residues in either TM II and/or VII. Additionally, the structural determinates involved in the dissociation rate of carbostyril **1** appear act indirectly to effect the dynamics of the interaction between carbostyril **1** and the receptor.

This study has been successful in many ways. There is data to further support the role of TM IV in the modulation of receptor activation by many agonists of different intrinsic efficacies. In terms of the mechanism of the subtype selective, slow dissociation of carbostyril **1**, the agonist-binding site has been shown analogous to the catecholamine-binding site using sophisticated complementary mutagenesis approaches that can be applied to other receptor problems. The structural features of carbostyril **1** that are required for slow dissociation from the β_2-AR has been elucidated and found to be a complex interaction between the 8-hydroxycarbostyril nucleus and salient features of the side chain. Additionally, the use of chimeric receptors has allowed the identification of a discrete region of TM IV, which contains four non-conserved amino acids, to be required for the slow dissociation of carbostyril **1**. Subsequently, the data was used to construct a detailed computer model of the complex between carbostyril **1** and the TM domains of the human β_2-AR. The model maintains the frog rhodopsin helical packing geometry and all structural features supported by experimental evidence following energy minimization and molecular dynamics simulations. Various structural features of the model suggest that TM IV is indirectly modulating the dynamics of the receptor to effect the dissociation rate of carbostyril **1**. The data suggest that carbostyril **1** slowly dissociates from the β_2-AR by a mechanism distinct from the exosite binding model proposed for the mechanism of salmeterol's long duration of action.

Several additional experiments could be performed to specifically address the hypothesis that TM IV is modulating the dynamics of the receptor to indirectly effect the dissociation behavior of carbostyril **1**. The first involves the direct measurement of conformational changes in the presence of carbostyril **1** using florescently labeled β_1AR and β_2AR. This approach was previously used to measure conformational changes of the β_2AR induced by ligands of various intrinsic efficacies, such as full, partial and inverse agonists (Gether and Kobilka, 1998). The use of florescently labeled β_1 and β_2ARs would allow the comparison of the conformational changes induced by carbostyril **1** without and with slow dissociation kinetics. The magnitude of the differences in the conformational changes may correlate with the dissociation rates of each of the carbostyril derivatives. This correlation would be related to the induced isomerization required for the slowest dissociation kinetics because all of the carbostyril derivatives are full agonists.

Additional molecular modeling with molecular dynamic simulations of the β_1AR, β_2AR, and various chimeric receptors with and without carbostyril **1** bound could identify differences in the helical movements upon ligand binding (Zhang and Weinstein, 1993; Luo *et al.*, 1994; Strahs and Weinstein, 1998). The approach could also be taken to simulate the binding of the additional carbostyril derivatives to attempt to correlate the dissociation rates with the helical motion induced in the β_2AR.

A project that could offer general knowledge to the GPCR field would be to perform site-directed mutagenesis on the four non-conserved amino acids in the fourth TM domain that appear to modulate the dissociation kinetics of carbostyril **1**. This would be to identify the residue most important for the difference in the dissociation behavior. The next step would be to restore the slow-dissociation of carbostyril **1** at the β_2AR

mutant by making complementary mutations in adjacent helices. If the approach is successful, the data would provide an additional inter-helical contact point that would accurately determine the inter-helical registry of TM IV with respect to other helices for future GPCR homology modeling.

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Stephen Todd Nowicki was born to Michael and Martha Nowicki on December 9, 1968, in Boynton Beach, Florida. After graduating from Frankfurt American High School, in Frankfurt am Main, Germany, he attended the University of Maryland, Munich Campus, Germany where he began to study chemistry. In 1987, he returned to the United States and continued at Florida Community College at Jacksonville, Florida. He transferred to the University of Florida and received a B.S. in chemistry in 1991. During his time at the University of Florida, Stephen began studying the functional selectivity of a family of streptococcal proteins called streptokinases while working with Dr. Richard Lottenberg. Stephen's interest in the structural determinates responsible for functional selectivity continued to grow while he continued to work with Dr. Lottenberg after his undergraduate studies. In 1992, he joined the M.D./Ph.D. program at the University of Florida College of Medicine. He began graduate studies in the Department of Pharmacology and Therapeutics at the University of Florida in July 1994. Following graduation, Stephen will complete the final two years of medical school and continue with residency training in medicine.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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